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TABLE OF CONTENTS

I. INTRODUCTION

Retinoblastoma is the most common malignant eye tumor of childhood and, after malignant melanoma, is the second most common primary intraocular malignancy of any type. From a clinical and pathological standpoint, retinoblastoma is unusual in several respects when compared with most other solid tumors and ocular tumors(4): 1) it tends to be multifocal and often bilateral; 2) there is a well established hereditary relationship in a significant number of cases; 3) it frequently occurs as a congenital tumor; 4) it not uncommonly undergoes spontaneous regression.

The tendency for retinoblastoma to undergo spontaneous regression has long been appreciated(68), and is well documented(52). The cause or causes for this occurrence in retinoblastoma have been speculated upon but careful histopathologic or other experimental investigation of the cause is lacking. The generally stated reasons for spontaneous regression are: 1) a sensitivity or other relationship to calcification; 2) the development of an immunologic response, suppressing the tumor; 3) the possibility of viral destruction of the tumor cells, possibly related to a viral etiology of the tumor itself.

The relationship to calcification is probably the most commonly cited of the various causes for spontaneous regression. The presence of calcium within the non-vitalized areas of the tumor is a striking clinical and histopathologic feature and also has in fact proved useful in the radiologic diagnosis of the tumor(52). This relationship has led Dr. F.H.Verhoeff to propose the use of calcifying agents, specifically vitamin D, as a means of treatment of retinoblastoma(97). This thisis presents a study

to determine if retinoblastoma cells are particularly more susceptible to calcification compared to other neoplasms in the tissue culture systems.

The possibility that spontaneous regression occurs on an immunologic basis is made questionable by several observations; in bilateral cases, spontaneous regression may occur in one eye and not the other. Even in various foci within the same eye, one may note one focus to regress whereas an adjacent and clinically similar area continues to grow. Moreover, frequently no inflammatory cell response has been found to be associated with the spontaneous regression. It must be borne in mind, nevertheless, that there have been few investigations regarding cell-mediated and/or circulating antibodies against the tumor in affected patients with retinoblastoma(21).

The occurrence of spontaneous regression of retinoblastoma has been compared to the spontaneous regression that occurs in certain virally caused lesions and this phenomenon has been cited as possible evidence that this tumor may have a viral cause(106). Evidence for a viral etiology of this tumor rests largely on the finding of reverse transcriptase(RNA directed DNA polymerase) showing a template specificity similar to that in oncogenis animal RNA viruses(72, 5) and experimentally induced tumors in animals using adenovirus(2).

Additional speculations regarding the cause for spontaneous regression exists. In 1956, Stewart, Smith, and Arnold suggested some unknown soluble factor that was responsible for this spontaneous regression(88). Wolter postulated that the tumor allows for little enlargement of the central retinal vessels feeding the tumor due to the dense fibrous ring in the lamina cribrosa that surrounds these vessels, thus limiting the vascular supply.

The clinopathological observations suggest that the regression of retinoblastomas appear to be associated with a relative avascularity of these lesions(14). Retinoblastoma contains a scanty stromal vasculature that are often tortuous and dilated, reflecting stasis and hypoxia. Consistent with this is the fact that when one wishes to induce a regression of a lesion in an eye by means of the laser or a light coagulator treatment destruction of the feeder vessels of the tumor seems the most effective way, far more effective in fact, than the attempted destruction of the mass of tumor cells themselves. This has led us to suspect that spontaneous regression of retinoblastoma may be the result of the lack of an adequate blood supply to the tumor cells.

Folkman and his associates have described in extensive detail the ability of tumors to stimulate neovascularization and the necessity of some factor initiating vascularization for the maintenenace of malignancies(17, 18; 34-36; 38-42;95). They have demonstrated that this substance is diffusable and have described various biological assays for measuring its activity. If tumor growth is not possible without such neovascularization, the development and administration of antibodies to this TAF may provide a new way of controlling tumor growth(34,36). If these findings are valid and apply to retinoblastoma, one might suspect that the spontaneous regression of retinoblastoma is the result of lack or deficiency, either by its intrinsic property of the tumor or by some inhibitory effect by the tumor on TAF.

This thesis is concerned with two aspects of spontaneous regression of retinoblastoma: 1) Retinoblastoma may lack TAF or has inhibitory factor to TAF and spontaneous regression occurs by vascular insufficiency; 2) Retinoblastoma cells undergo necrosis with an increased susceptibility when the calcium concentration is elevated.

II. Retinoblastoma

1. Epidemiology and Genetics;

The obscure first documentation of retinoblastoma may trace back to 1597 when Petrus Pawium of Leyden, Holland, made an autopsy description of the tumor as "filled with a substance similar to brain tissue mixed with thick blood and like crushed stone"(14). During the early nineteenth century, it was referred to as "fungus hematodes" and since then it has been known as glioma or neuroepithelioma of retina. It was in 1864 when retinoblastoma was first fully described by Virchow. Its present name was given by Verhoeff in 1922.

Although it constitutes only 0.01 per cent to 0.4 per cent of all ocular disease, retinoblastoma remains a severe disease; 19 per cent of the affected cheldren still die from it in the U.S.A. and about one per cent of the deaths from cancer in early childhood is due to this tumor(37).

Its incidence is between one per 15,000 and one per 35,000 and most cases occur before age three and are unusual after age seven, although few are seen in middle ages. There is no sex predisposition but is slightly more common among Caucasians.

Retinoblastoma has been seen with increasing frequency, but the familial nature of the tumor was not recognized until the latter part of the nineteenth century. There are two ways in which an individual is hypothosized to have the genetic material for retinoblastoma; the gene may be transmitted by a parent(germinal mutation) or may be acquired as a result of mutation(somatic mutation). A strong family history is often associated with bilateral disease which occurs in 35 per cent of the cases. The disease occurring as a

result of somatic mutation is expected to be unilateral (14). The familial retinoblastoma are believed to represent a dominant autosomal gene that exhibits variable penetrance (9). However, many observations speak against a simple genetic cause (14): a lack of complete expressivity, as shown by the absence involvement of total retinae and concurrence of structurally and functionally normal retinae in the eye harboring the retinoblastoma; a differential involvement and progression of the tumor in the same patient; a lack of histological difference between unilateral sporadic retinoblastoma from bilateral familial retinoblastoma (106).

A sporadic unilateral case will transmit the disease to 10-20 percent of his children while a sporadic bilateral case will pass it onto fifty percent of his progeny (28). Some authors feel that those with strong family history especially if the tumor is present bilaterally should avoid having children (14).

Stallard and Penrose reported a chromosome deletion in one individual with retinoblastoma (86). Others have described a high incidence of retinoblastoma in D-deletion syndrome and in Trisomy 21. However, it should be noted that mental retardation is not observed in the majority of patients with retinoblastoma (37). A biochemical search for the tumor diagnosis made by measuring vanillylmandelic acid and homovanillic acid excretion has also been inconclusive.

2. Histopathology

Retinoblastoma cells were thought to be "bipotential" cells

that could follow the neuroblastic series to form neurons or the spongioblastic series to form glia (92). However, the evidence that retinoblastoma cells are neural in origin is provided by T'so, Fine, and Zimmerman by histopathological as well as electronmicroscopic studies (93,94): retinoblastoma cells were demonstrated to produce structures resembling photoreceptor elements without formation of Muller cells. Since the formation of Muller cells is an evidence of neuroglial differentiation, the observation of photoreceptor differentiation as shown by rosettes and fleurettes without accompanying formation of Muller cells indicates that the retinoblastomas are neuronal neoplasms rather than gliomas as Virchow and others assumed (94).

The Flexner-Wintersteiner rosettes are the morphological hallmarks of retinoblastoma differentiation and, as described by T'so and others, represent a differentiation of tumor cells toward retinal photoreceptors (93); these rosettes are made up of uniform cuboidal cells arranged in an orderly fashion around a small lumen with nuclei at the base away from the lumen. The ultrastructural appearance suggests a differentiation of rods and cone-like structures from the outer nuclear layer-related cells. A more differentiated form of tumor cells resembling a bouquet of flowers or fleurettes have also been shown to produce photoreceptor elements (93).

The normal histological picture of rods and cones consists of an outer segment composed of several layers of discs

that are connected to the inner segment by a cilium that characteristically contains nine microtubules arranged in a circular pattern around a center that is devoid of tubules. This is what the electron microscopists call the 9+0 pattern that serves as an identifying feature of the photoreceptors that is also seen in the rosette cells of retinoblastoma (94). A further evidence that retinoblastoma cells are potential photoreceptor elements comes from a tissue-culture study by Albert and coworkers (3). Among other cellular elements, microtubules are present in photoreceptor cells, bipolar cells, horizontal cells, ganglion as well as Muller cells. Usually the microtubules of other cell origin are demonstrated only when fixed with gluteraldehyde but the microtubules in the axons of photoreceptor cell line are preserved in chrome osmium fixed tissue as well as in gluteraldehyde. The observation that the microtubules of retinoblastoma cells directly from the tumor as well as from the suspended culture are demonstrable by both means suggests that retinoblastoma cells in vivo are related to photoreceptor elements (3). The less well differentiated retinoblastoma cells resemble immature embryonic retinal cells showing a rather uniform chromic nuclei and scanty cytoplasm with abundant mitotic figures. This histological picture is difficult to differentiate by light microscopy from many other anaplastic small-cell neoplasms such as neuroblastoma of adrenal gland, cerebellar medulloblastoma, and oat-cell carcenoma of the lung (28).

3. Metastasis:

Multicentric sites of origin are common in approximately 60 to 84 percent of tumors (28). Such multiple sites may reflect either true multicentricity in various areas of the retina or a tumor seeding in the vitreous to retinal surface. The invasion of optic nerve is common, but there is no particular area of fundus for which retinoblastoma has a predilection but as many as 75 percent of cases involve the ora serrata either as a part of massive involvement or as small localized tumors. This probably reflects a mechanical trapping at this location as tumor cells are spread in the subretinal space. There is a clinical variant in which the entire retina is uniformly replaced by the tumor cells without forming a prominant tumor mass. This is referred to as diffuse infiltrating retinoblastoma, and is more apt to invade the uvea and extraocular region that other types (28).

Histopathologically, retinoblastoma characteristically lacks a well-developed stroma with the tumor cells adhering to one another loosely, and necrosing is a common feature. This may, in part, account for the intraocular spread to various sites. As tumors grow larger within the eye, the outer portions tend to break away from its parent tumor body and seed the vitreous cavity. This is considered to be a bad prognostic feature despite the lack of significant supporting vasculature in these tumors and the generally slow growth rate (28). The tumor may spread by a direct invasion to

the optic nerve substance or by extension to the ciliary body, iris, chamber angle, and corneal endothelium, and metastasize via the optic nerve and blood vessels. Extraocular extension and systemic metastasis of retinoblastoma occur most commonly through the optic nerve and scleral emissary veins. Tumor cells directly seed the subarachnoid space via the cerebral spinal fluid. Another important route of metastasis is an extension through the uvea into the blood stream. The most frequently involved site in the early stage of metastatic involvement is the bone marrow. Other commonly involved organs are lymph nodes, liver, and spinal cord. No organ of the body is immune to retinoblastoma metastasis (9), although it is never seen in the lung except by a direct spread. The characteristic appearance of rosettes are not seen in metastasized retinoblastoma (28). It is difficult, therefore, to make the diagnosis from the examination of the metastases, if the diagnosis has not been established previously.

4. Diagnosis:

The early clinical diagnosis of retinoblastoma depends on the observer's index of suspicion. The most common presenting sign of retinoblastoma is a "white pupil" or "cat's eye reflex". If the tumor involves the macula region, the white reflex will be present when looking straight ahead, however, if the tumor arises in the retinal periphery it is seen only when looking in a certain direction, thus frequently delaying the diagnosis considerably (28).

The second most common presenting sign of retinoblastoma is strabismus with esotropia showing greater preponderance over exotropia. Again it is an early sign if the tumor involves the macula region. Therefore any child with strabismus with poor fixation should be considered as having retinoblastoma until proven otherwise (82). Inflammation characterized by a red painful eye with or without glaucoma as well as hypopyon and hyphema is also not an uncommon clinical presentation. A violent inflammatory response resembling endophthalmitis panophthalmitis, or orbital cellulitis may be seen. Nystagmus may be a manifestation of an advanced bilateral disease. Other signs include squint, mydriasis, and darkening of iris secondary to bleeding and hemoglobin deposition.

Histologically, two features may be considered suggestive of retinoblastoma; presence of calcification and its multifocal appearance. Calcium is detected in fifty percent of retinoblastomas and often a useful roentgenographic sign of the disease. Calcium on the tumor surface is sharply demarcated and has a cottage-cheese like appearance, while the calcium within the tumor is seen as a gray colored nodule with non-distinct outlines. However, some white glistening mass resembling calcified foci have been shown to be precipitated DNA material rather than calcium (26). Calcification may also occur in tuberculosis, ocular syphillis, toxoplasmosis, sarcoid, Coat's disease and parasitic infections such as

cysticercosis and should be differentiated from that of retinoblastoma (14).

Retinoblastomas should be differentiated from conditions that produce "leukokoria" or white pupil. Cataract formation is not often a part of the picture of retinoblastoma. Some conditions, that may be confused with retinoblastoma are larval granulomatosis that presents as a relatively quiet subretinal nodule or uveitis or endophthalmitis with vitreous opacification, and retinal astrocytoma. Of those lesions that are not visible, granulomatous uveitis of any cause, Coat's disease (that typically shows aneurysmal vessels in the retina), metastatic retinitis, persistent hyperplastic primary vitreous (PHPV), retrolental fibroplasia, vitreous hemorrhage, colobomas, medullated nerve fibers and massive stasis should be differentiated from retinoblastoma. Moreover, retinoblastoma occurs in the eyes of normal size and is rarely seen in microphthalmos (28). The persistent hyperplastic primary vitreous was the most frequent of conditions other than retinoblastoma causing leukocoria in a series of five hundred cases by Howard and Ellsworth. An important differentiating feature is the presence of long ciliary processes around the periphery of the lens, shallow anterior chamber, and microphthalmic eye in PHPV.

The most useful diagnostic tool for retinoblastoma is an ophthalmoscopic examination that, if the media is clear, can easily spot the more common endophytic type; it arises from

the inner nuclear layer, then the nerve fiber layer, or the ganglion cell layer extending into the vitreous cavity as a pinkish creamy mass with surface neovascularization, or as a white chalky mass (14). An exophytic tumor that arises from the external nuclear layer has the characteristic appearance of a retinal detachment. A patient with suspected retinoblastoma should be examined under anesthesia with indirect ophthalmoscopy and indentation for visualization of the peripheral retina.

A biochemical study may be helpful in diagnosing retinoblastoma; Swartz and Goldberg observed a markedly increased LDH (lactic dehydrongenase) in the aqueous humor of an eye harboring the retinoblastoma (89). LDH is presumably released by the tumor cells as they undergo necrosis. LDH level was higher by fifty percent compared with serum level and was distinguished from only slightly elevated levels observed in other disorders such as endophthalmitis and malignant melanoma. Although not sufficient as definitive a diagnostic tool by itself, this simple method may be helpful as adjunct in the diagnosis of retinoblastoma, one should keep in mind the danger of tumor spread associated with paracentesis.

However, the misdiagnosis of retinoblastoma may be as high as 14.9 percent (87). This may in part be due to the presence of vitreous opacity, cataractous changes, or cloudy cornea that will interfere with a direct visualization of the

intraocular process. A recent introduction of B-Scan ultrasonography may be a significant diagnostic aid in cases where visualization by ophthalmoscopy is impaired or if radiographs fail to show any calcification (87). It may especially be helpful in following a patient who has vitreous hemorrhage complicating the radiation therapy of retinoblastoma and tumor mass cannot be visualized. However, if the scan reveals a cystic rather than solid acoustic pattern, an organized hemorrhage in the vitreous may be indistinguishable from a tumor.

Roentegenography may be helpful, since about 75 percent of retinoblastoma show nonosseous calcium. The usefulness of fluorescein angiography is controversial. A cytologic examination of material aspirated from the anterior chamber may be helpful, when a recurrence of retinoblastoma is suspected in the fellow-eye many years (8-12 years) after a successful treatment of retinoblastoma in the other eye. Yttebort and Arnesen believe that the late recurrence of retinoblastoma may present as an inflammatory condition and finding of granulocytes in a sterile aqueous humor favors a neoplastic rather than an inflammatory lesion (103). Other laboratory measures such as peripheral smear, bone marrow aspiration and biopsy, spinal tap, and urinary VMA should be considered both for diagnostic as well as therapeutic purpose.

5. Prognosis:

Microscopically the retinoblastoma may show an endophytic

and/or exophytic pattern of growth; the former protrudes inward toward the vitreous from the retina, and because of its protuberance above the retina, is more easily detectable with an ophthalmoscope than a subretinal tumor. Such endophytic growth, however, is more likely to seed the vitreous which severely worsens the prognosis. Exophytic growth occurs in an outward manner toward the subretinal space and is more difficult to diagnose because of its resemblance of simple retinal detachment (28). Consequently, exophytic type tends to be diagnosed at a more advanced stage. Most tumors, however, show both types of growth. In determining prognosis, one should consider that the secondary changes such as inflammation, hypoypon, hyphema, closed angle glaucoma, as well as complication of therapeutic measures. For example, it is not uncommon to see hemorrhage after treatment either with radiation or light coagulation. Approximately ten percent have an atrophic annulus due to retinal pigment epithelium disruption as the border of the tumor that has been interpreted as a sign of regressive stage and therefore a favorable sign (28). Although calcification is often interpreted as a reactive degeneration of necrotic foci, some retinoblastoma may occur clinically as snow-white, elevated, avascular mass that does not imply a benign course such as a vascular tumor is seen more often in older children of ages six and eight and arises in the retinal periphery where it grows slowly and produces retinal detachment and

subsequently visual loss.

In general, the less differentiated retinoblastoma occurs with larger tumors, tends to invade the optic nerve, and indicates a more virulent course, despite the greater tumor cell sensitivity to radiation (28). It should be emphasized that factors other than cell type have significant role in determining the prognosis of retinoblastoma. They are (1) the size of the tumor, (2) location of the tumor involvement, and (3) the degree of tumor invasion of the optic nerve or subarachnoid space or both (9). Of these the invasion of optic nerve is the most ominous with regard to prognosis. It is not uncommon to see a rapid growth leading to metastasis and death in retinoblastoma with a histological picture showing a more differentiated cell type characterized by many rosettes. Conversely, a tumor composed of entirely undifferentiated cells may be more curable. In terms of treatment, a more differentiated tumor may be more refractory to radiation therapy as it is less sensitive.

Redler and Ellsworth too, emphasized the size and location rather than the cell type of the tumor in determining the prognosis. A clinical classification of retinoblastoma has been formulated by Ellsworth and has been widely accepted:

Group 1 (very favorable)

a. Solitary tumor, less than 4 dd in size at or behind the equator
b. Multiple tumors, none over 4 dd in size, all at or behind the equator

Group 2 (favorable)
a. Solitary lesion, 4-10 dd in size, at or behind the equator
b. Multiple tumors, 4-10 dd in size, behind the equator

Group 3 (doubtful)
a. Any lesion anterior to the equator
b. Solitary lesions larger than 10 dd behind the equator

Group 4 (unfavorable)
a. Multiple tumors, some larger than 10 dd
b. Any lesion extending anteriorly to the ora serrata

Group 5 (very unfavorable)
a. Massive tumors involving over half the retina
b. Vitreous seeding

Solitary or multiple small lesions at or posterior to the equator have a favorable prognosis, while the large multiple tumors involving the area anterior to the equator and vitreous signify a poor prognosis. The poor prognosis with an anterior involvement stems from the fact that it is frequently missed by a radiation beam that is directed to avoid the lens and is frequently overlooked. Furthermore, the anterior part of the globe is an area where it is difficult to apply light coagulation without complication (26).

The invasion of optic nerve greater than three mm posterior to the nerve head raises the mortality rate to 60 to 70 percent or higher, compared to only 10 percent in patients whose tumor does not involve optic nerve or emissaria and has not seeded into the vitreous (8). Scleral extension of the tumor has about 70 percent mortality, while full thickness choroidal involvement signifies 60 percent mortality (28).

The involvement of choroid per se does not signify a very poor prognosis. Although in some studies choroidal invasion has been associated with high incidence of metastasis and fatal outcome, Redler and Ellsworth, by serially sectioning all enucleated globes, found the volume of choroidal invasion rather than the presence of choroidal invasion to have the prognostic significance. The importance of choroidal involvement lies in the fact that blood borne-metastasis may result from invasion of choroidal circulation, which predisposes to hematogeous spread. The involvement of the choroid may be heralded by a rapid growth over a period of days or weeks, high elevation of narrow, pedunculated stalk, and a yellow color as lamina vitrea is pushed forward (70).

Therefore, the practical factors that reflect the prognosis include rapidity of diagnosis, size or mass of the tumor, degree of vitreous and anterior chamber seeding, degree of optic nerve involvement, and resectability of the tumor (9). It should be noted that the prognosis is not significantly worse in bilateral cases; the more extensively involved eye is enucleated while the fellow-eye is subjected to local treatment (28). There seems to be a correlation between prognosis and the degree of tumor involvement in the eye with a larger mass.

6. Treatment:

During the early nineteenth century, retinoblastoma was treated with various combinations of leeches and cupping,

unctions, vesicants and purgatives, and incision and drainage of the globe (14). Although James Waldrop of Edinburgh suggested an enucleation of the eye with retinoblastoma, the potential of neoplastic nature of retinoblastoma was not widely appreciated until the mid-nineteenth century. Attention then was directed to early diagnosis and therapy with preservation of vision in bilaterally involved cases. In early twentieth century, radiation was first used by Hilgartner with a good result in conserving a useful vision. The next significant technical advance occurred in 1953, when combination therapy of mustard such as triethelene melamine (TEM) and radiation therapy was first employed by Dr. Carl Kupfer. Since then refinements of ionizing radiation and chemotherapy along with photocoagulation and cryotherapy improved the prognosis for life and vision. The overall survival rate in 1869 was 5 percent as compared to 81 percent in 1967 (14). The most important factor in improving the survival rate was the early diagnosis and enucleation.

The most significant factor regarding the effectiveness of treatment of retinoblastoma is the stage of the disease at the time of treatment is undertaken (10). It depends whether it is confined to the eye, has invaded the orbit or has metastasized. Prior to treatment, patients should be evaluated for systemic metastasis which includes roentgenograms of the skull, orbits, and optic canal; a skeletal survey with bone scan; bone marrow aspiration and biopsy at one or

more sites; and a cerebrospinal fluid examination for tumor cells. Having mapped out the regions of tumor involvement as precisely as possible, the therapy may be divided into two categories: treatment of unilateral or bilateral tumor. A surgical excision of the tumor is not favored once the metastasis has occurred.

The unilateral retinoblastoma is usually treated by enucleation. Except in an instance where tumor arises in the macula and produces a squint that leads to an early diagnosis, the most unilateral cases (approximately 65 percent) at the time of discovery and diagnosis show a tumor of considerable size. However, Ellsworth suggests that small tumors falling into his clinical groups 1, 2, and possibly 3 be treated nonsurgically. In usual bilateral retinoblastomas where one eye is more extensively involved than the other, the more advanced eye is generally enucleated while the other is treated in an attempt to preserve vision (28).

Reese and his coworkers advocate radiation alone as the primary mode of treatment in groups 1, 2, and 3, and radiation with intracarotid TEM 0.1 mg/kg in groups 4 and 5. An average dose being given is about 3500R in three weeks, for doses greater than 5000R may produce vascular necrosis of particularly radiosensitive retinal and choroidal blood vessels (28). The greatest concern in these radiated patients is the increased incidence of acquiring second primary tumors, especially osteosarcomas, fibrosarcoma and

chondrosarcoma. Other complications include changes in retinal pigment epithelium especially when detached, glaucoma, radiation dermatitis, cataract and atrophy of bone over the temporal area (86). The treatment of recurrences with a second dose of irradiation is not indicated because of low salvage rate (15 percent) and because of increased incidence of severe intraocular hemorrhage and glaucoma. An examination under anesthesia is performed 6-8 weeks later and light coagulation or CO^{60} application is used to treat any residual tumor (14). Despite a continued need for a close supervision, a treatment may be considered a success if the tumor appears inert for six to nine months in the area where the therapy was administered. However, a continued close supervision is necessary. A palliative measure in metastatic retinoblastoma has been chemotherapy; triethylene melamine, cyclophophymide, vincristine have been tried with less than satisfactory results due to inevitable side effects such as hematological depression and infection (14). Nitro sources SCNU, CCNU are recently being considered for patients with CNS metastasis. Other valuable adjuncts in the management of retinoblastoma, especially for the small lesions or local recurrences, have included light coagulation, cobalt applicators, radon seeds, diathermy, and cryotherapy.

With this background on retinoblastoma, the phenomenon of spontaneous regression seen in this neoplasm will be discussed in greater detail.

III. SPONTANEOUS REGRESSION OF RETINOBLASTOMA: THE POSSIBLE ETIOLOGIES

The phenomenon of spontaneous regression seen in retinoblastomas is an unusual occurrence rarely noted in other malignant neoplasms. A prominant amount of calcification is noted in all spontaneously regressed tumors. Eyes that contain regressed retinoblastoma, often show phthisis bulbi--an atrophied shrunken eye with extreme disorganization, and fibrosis which may become hyalinized and calcified. This may represent a reactive degeneration of ocular tissues to necrotic tumor cells. Such shrunken eyes are often sightless; Anderson and Jensen demonstrated phthisis bulbi in all but one of fourteen spontaneously regressed retinoblastomas (7). Boniuk and Girard concluded that an eye that has been phthisical since childhood should be suspected of harboring regressed retinoblastoma (15). The presence of areas of calcification with remnants of pyknotic nuclei lying adjacent to areas of ossification is pathognomonic of regressed retinoblastoma. Boniuk and Zimmerman reported fourteen cases of regressed retinoblastomas (16), again the striking feature was the presence of large nests of calcified tumor cells within the area formally occupied by the retinal and vitreous body. With one exception, eyes containing regressed tumor were phthisical. However, not all retinoblastomas in phthisical eyes were completely regressed. Thus the presence of endophthalmitis or phthisis bulbi in a young child should raise the suspicion of an underlying retino-

blastoma that may,or may not, have undergone regression. Any patient with ophthalmoscopic picture of a regressed retinoblastoma is a candidate for recurrence and should be followed thereafter for an early detection of possible tumor involvement in the fellow-eye.

Although the ability of retinoblastoma to undergo spontaneous regression has long been appreciated and is well documented, the cause and pathogenesis of this phenomenon have not been clarified. The generally cited causes of spontaneous regression are: (1) a sensitivity or some other relationship to calcification, (2) development of immunological response, (3) viral transformation, and (4) vascular insufficiency.

1. Role of Calcium:

A striking clinopathological feature of a regressed retinoblastoma is an almost invariable presence of calcification in the necrotic foci, and this had led one to suspect some etiologic relationship between calcification and regression. Although the calcification of tumor cells may represent a secondary degeneration due to ischemia and necrosis, the possibility of a particular vulnerability of retinoblastoma cells to calcification is still present. When other tumors undergo regression or have obstruction of their blood supply, calcification is not seen (88). For instance, sympathetic ophthamia and melanoma, two common causes of ocular degeneration, do not lead to calcium deposition.

Not a single instance of calcification among 100 globes removed because of sympathetic ophalmia and 84 globes containing melanoma is reported (105). No reports of a controlled series utilizing such treatment or any in vitro study on calcium sensitivity have been reported. One may be able to test the possibility of calcium sensitivity in vitro by adding higher concentrations of calcium to the tissue culture cells of retinoblastoma and also comparing the behavior of other tumor cells in vitro under same conditions.

2. The Role of Immunological Response:

In most other human malignancies where spontaneous regression has been observed, such as human malignant melanoma and neuroblastoma, it has been noted that patients with a given histological tumor cell type has had either autologous or allogeneic immunological reactivity against that tumor type, suggesting an immunological mechanism may well be responsible for humor regression (21). A support for this idea comes from a study by Char and coworkers of cell-mediated immunity to a retinoblastoma tissue culture line in patients. In comparison to control groups, patients with retinoblastoma had a significantly increased lymphocytotoxicity against the retinoblastoma cell line as measured by the ^{125}IUDR cytotoxicity assay. Also, crude membrane extracts injected as skin tests produced delayed hypersensitivity in patients with retinoblastoma but not in those without retinoblastoma (14).

If such immunostimulation is definitive, one may even be able to correlate the presence of reactivity to the retinoblastoma cell line in vitro with the disease status or prognosis (21).

The nature and specificity of retinoblastoma tumor antigen is not clear. The presence of some shared antigen related to retina is a distinct possibility, since patients with pigmentary retinal degenerations of various types, which frequently begin in the photoreceptor layer, are shown to have a significantly greater cytotoxicity than the control normal subjects against the cell line of retinoblastoma. The patients with other kinds of retinal abnormality did not have increased levels of cytotoxicity (22).

The claim that spontaneous regression occurs by an immunological mechanism is disputed by several observations: In bilateral cases, spontaneous regression may occur in one eye only with growth of the tumor and orbital invasion in the other eye. Even in various foci within the same eye, one may note one focus to regress whereas an adjacent and clinically similar area continues to grow. Moreover, no inflammatory cell response has been found to be associated with the spontaneous regression in the majority of retinoblastomas.

3. Role of Viruses:

Viral etiology in spontaneous regression of retinoblastoma has also been considered recently. Kobayashi and Mukai (57)

using intravitreal injections of adenovirus type 12 in hamsters and mice, reported the production of retinal tumors resembling neuroepitheliomas. The tumors formed perivascular rosettes and had cellular 9+0 tubular pattern.

Furthermore, since the original hypothesis of Temin that induction of cancer by RNA viruses requires an enzyme capable of making DNA from RNA templates i.e. RNA reverse transcriptase, over twenty-nine different RNA tumor viruses have been shown to contain such an enzyme (2). The fact that viruses are capable of producing tumor cells possessing virus specific enzyme suggests the possibility of testing retinoblastoma for the presence of such virally produced substance. Since RNA-directed DNA-polymerase is found to be present in all of the animal RNA tumor viruses analyzed, one may be able to use this enzyme as a "footprint" of the tumor virus, since it is possible that enzyme and the DNA which code for the virus could be present without being present as an identifiable morphological identity (71).

An experimental clue to the basic cause of retinoblastoma may lie in the observation of RNA directed DNA-polymerase activity with a template specificity similar to RNA oncogenic viruses (5). Albert and Reid were able to assay ten specimens of retinoblastoma for reverse transcriptase and show RNA directed DNA-polymerase activity in an overall pattern exhibited by RNA tumor viruses. They also compared such specific enzyme activities in the tumor region, in the retina adjacent to the tumor, and in the retina distal to the tumor

and found that the greatest enzyme activity is found in the tumor region: The farther away the area from the tumor, the less the activity of the enzyme (2).

4. Tumor Angiogenesis Factor:

The uninvestigated but potentially important cause of spontaneous regression of retinoblastoma is the possibility of vascular insufficiency. Folkman and his associates propose that "tumor neovascularization is the result of a specific humoral stimulus secreted by the tumor--to elicit new capillaries from the host and that these vessels, in turn, become an important control mechanism in the growth of tumor"(35). A lack of such a substance and resultant vascular insufficiency would prevent a continued tumor growth. However, the existence of tumor angiogenesis factor (TAF) and its role in the spontaneous regression of retinoblastoma are not established. TAF is described to be a non-histone protein substance with molecular weight of approximately 100,000 that is vulnerable to ribonuclease or proteases and heat to 56 degrees centigrade, but unharmed by trypsin. It is composed of 25 percent RNA associated material, 10 percent protein, 50% carbohydrate with a lipid component which tends to decrease TAF activity and if removed, restores the TAF activity (95).

Although most work by Folkman and associates have recently focused attention on this area, the presence and role of blood vessel forming factor in relationship to tumor growth has been

a source of speculation for a long time. In the following pages are some of the recent works showing (1) a continued tumor growth requires a continuing vascular supply to the tumor, (2) tumor elaborates a tumor-specific angioproliferative factor (TAF), and (3) TAF may be an expression of tumor malignancy.

In early 1900s neuropathologists observed and described the patterns of microvasculature found in brain tumors. Although it was dismissed as a host reaction to growing tumor, there has been a growing evidence to show that there is a specific and direct ocrrelation between the tumor growth and vascular supply to the tumor. Although most effort in regard to tumor growth has been directed toward understanding the biology of neoplastic cells themselves, the study of the environment in which these tumors reside may hold significant promise as observed by Hardman in 1940:

> It was natural that attention should be focused on the cellular elements of gliomata rather than the vessels--- and yet these vessels tell a story would it be read (50).

Folkman and Hochberg recently observed that,without vascular supply, a persistent growth of tumor cells occur only in two dimensions, as in tissue culture, but not in three dimensions, as in solid tumors (33). They observed that an isolated spheroid of tumor cells eventually reached a maximum mean diameter beyond which a further expansion

was not possible, regardless of frequency of replenishment of the medium. B-16 mouse melanoma, V-79 Chinese hamster lung and L-5178 murine leukemia cells, all grew as spheroids but eventually reached a dormant phase at the diameter of approximately 3-4 mm and a population of approximately 10^6 cells. This dormant phase is defined as a state of equilibrium between the newly generated cells at the periphery balanced by those lost by necrosis in the center. They postulated that the dormancy is secondary to a reduction in the ratio of surface area to volume and consequently an inefficient elimination of catabolites and absorption of nutrients. These authors provide a mechanism for in vivo solid tumor growth: "before vascularization, solid tumors live by simple diffusion as three dimensional spheroids or ellipsoids. They become dormant at the diameter of only a few ml; once vascularized they are released from this dormant phase and begin exponential growth. Therefore, tumor dormancy resulting from the absence of angiogenesis in vivo may operate by the same mechanism for dormancy of spheroids in vitro"(33). The histology of these dormant tumors typically shows a central necrotic zone, a viable mid-zone, and an outer mitotic zone. By using thymidine labelling as the mitotic index of the mouse mammary tumor, Tannock showed a direct relationship between the proximity of cells to blood vessels and their mitotic index (90). The closer the cells were to the vascular supply, the greater the mitotic index and the lesser the degree of necrosis.

Brem and Folkman also found that the degree of capillary proliferation was related to the extent of growth of brain tumors (17). Tumors placed in the avascular compartment such as the anterior chamber persist for a prolonged period as small but viable dormant nodules. Greene maintained a human glioblastoma in the anterior chamber for nine months without change in size or metastatic spread (48). In isolated perfused organs where endothelial cells had degenerated and neovascularization was not possible, tumor implants were unable to grow greater than the size of 2-3 mm diameter.

Furthermore, tumor growth can be deliberately arrested by preventing vascularization. Gimbrone, Folkman, and others showed that Brown-Pearch epitheliomas implanted in the iris of male New Zealand white rabbits were able to undergo exponential growth phase once vascularization occurred, but the same tumors placed within the anterior chamber out of contact with the iris remained dormant at a small size and yet were able to undergo the same rapid growth phase if transplanted to iris where vascularization was possible (38).

Evidence for a tumor specific induction of host vascular proliferation is claimed by Feigin, Allen, Lipkin and Gross (32) who reviewed 433 human tumors involving the brain and found that both the primary brain tumors and metastatic carcinomatous nodules showed marked endothelial hyperplasia. This suggested that a specific communication between the

tumor and host vessels was not influenced by the type of tissue surrounding the tumor. It has been observed also that the nearer the tumor is placed to the source of host vascular supply the faster the initiation of endothelial proliferation and vascular formation (55).

A strong correlation was made between the degree of endothelial hyperplasia and the degree of histological malignancy as evaluated by cellularity, variability in cell size and shape, and hyperchromatism of the cell nuclei (39). Gimbrone and Bullino suggested that TAF production may be a reflection of neoplastic transformation, since the ability to induce neovascularization seems to be quantitatively correlated to the degree of malignancy (42). Algire and Chalkley noted that, given the same distance between tumor implants and the host vessels, tumor fragments induced the neovascularization much earlier and more vigorously than normal tissue grafts or wound (6). Ide, Baker, and Warren made similar observations; tumors induced a vascular growth in approximately three days, in contrast to the six days required by normal tissue (55).

Folkman proposed that solid tumors have an avascular phase. The initial avascular phase occurs when growth is not appreciable and remains at a small size. This phase is followed by a vascular phase characterized by the penetration of the host vessels into the tumor substance with a resultant exponential growth of the tumor (39). According to Folkman,

it is the TAF that initiates the vascular phase, and since the vascular phase is associated with the malignant properties that a tumor displays, it is possible that one may be able to prolong the avascular phase by producing an anti-TAF, thus arresting the tumor growth and making the tumor more vulnerable to other conventional modes of therapy.

One difficult aspect that still remains to be clarified is to distinguish the tumor angiogenesis from other processes that lead to neovascularization such as inflammation, wound healing, and delayed hypersensitivity. Since tumor implants may inevitably produce some inflammation one can argue that neovascularization of the tumor is secondary to 1) inflammation and wound healing, or 2) immune response to tumor cells, rather than TAF mediated neovascularization (4). An inflammatory response followed by neovascularization is seen in both wound healing and tumor implantation. The major difficulty lies in differentiating the neovascularization induced by TAF from that induced by an inflammatory process.

The role of inflammation in angiogenesis may be studied by producing a corneal injury in rabbits whose functional immune system has been obliterated by radiation or by anti-lymphocytic serum (ALS). Observations have been made that corneal neovascularization, following a corneal silver-nitrate burn, did not occur in rats receiving total body irradiation (ARVO meeting, 1975, Klintworth). The vascularization induced by an immune response is thus apparently eliminated by radia-

tion. Attempts have been made to distinguish the tumor induced capillaries from the inflammation induced capillaries on the basis of morphological differences. Some observers have noted that tumor induced vascularization, when compared to nonspecific inflammation-induced vessels, was 1) initiated earlier, 2) proliferated for a longer period of time, 3) had a greater virulence, and 4) did not have any differentiation of capillaries into arterioles and venules (66).

Gimbrone et al. (39) observed that typical tumor vasculature shows 1) an increased density of small vessels and the presence of endothelial mitosis in histological sections, 2) greater ^{3}H-thymidine-labelling of endothelial cell nuclei in capillaries, and 3) a distinctive pattern of small vessel formation characterized by marked tortuosity and "corkscrew formation" suggesting that the endothelial proliferation resulted in an elongation of existing vascular channels rather than an outgrowth of new capillary sprouts. Despite these claims, on microscopic examination, tumor specific vasculature is often very hard to differentiate from inflammatory vasculature.

The presence and the role of TAF in retinoblastoma is unknown. It is the purpose of the following study to see if such an angioproliferative factor can be demonstrated in retinoblastoma and whether or not this TAF may have a role in its spontaneous regression.

IV. Tumor angiogenesis factor; an experimental study.

1. Introduction.

Solid malignancies in man and animals are dependent upon a vascular supply from the host for sustained growth. It has been generally assumed that the tumor cells need contact with capillaries arising from the host, since they can not provide their own capillaries. It has been postulated that the supportive vascular response following tumor implantation is the result of interaction between the tumor and the host organism and is mediated by the tumor(17, 18, 34-36; 38-42; 94). It was the purpose of the following series of experiments to test whether in retinoblastoma such an angioproliferative factor could be demonstrated and, if so, to attempt to compare it quantitatively to that of other ocular tumors. In addition, experiments were designed to evaluate possible factors that might have a role in the spontaneous regression of retinoblastoma. The study was done in four parts:

A) The corneal implantation of non-tumor substances:

Exp. 1A. the effect of creating corneal pockets alone.

Exp. 1B. the effect of injecting formulin into the corneal pocket.

B) The corneal implantation of viable tumors:

Exp. 2. the effect of corneal implantation of viable retinoblastoma and melanoma cells derived from the tissue culture growth.

Exp. 3. the effect of corneal implantation of viable retinoblastoma and melanoma implants derived from the solid human and animal tumors.

C) The corneal implantation of treated tumors:

Exp. 4A. the effect of corneal implantation of retinoblastoma implants and melanoma implants that have been fixed in 10 per cent phosphate buffer.

Exp. 4B. the effect of corneal implantation of retinoblastoma and melanoma clumps that have been fixed in 10 per cent phosphate buffered formalin and washed in normal saline.

Exp. 5. the effect of corneal implantation of retinoblastoma and melanoma implants that have been boiled in normal saline.

Exp. 6. the effect of corneal implantation of retinoblastoma and melanoma implants that have been fixed in gluteraldehyde.

D) The corneal implantation of viable tumors in treated host:

Exp. 7. the effect of intraperitoneal and topical injection of indomethacin proir and following the implantation of viable tumors in rabbit corneas.

Exp. 8. the effect of corneal implantation of viable retinoblastoma and melanoma clumps in rabbits treated with an immune depressing dose of radiation(approximately 900R).

2. Materials and Methods.

Animals; New Zealand albino male rabbits weighing 1.5-3.0 kg. were used in these experiments. Each rabbit was kept in an individual cage and maintained at normal room temperature. Routine feeding and daily care were provided.

Tumors; Retinoblastoma was obtained from the following sources:

1. an established line retinoblastoma(Y-79) and (238-1) from tissue culture culture growth(73).
2. solid human retinoblastoma; uniocular retinoblastoma enucleated from a ten month old white male from a ten month old white male from Columbia-Presbyterian Hospital.
3. one of the bilateral congenital retinoblastoma removed from a three month old male (white) from Oswego, N.Y. There was no known family history of retinoblastoma in either patient.

Melanoma was obtained from the following sources:

1. Hamster-Greene melanoma from tissue culture growth(22).

2. Dog melanoma, spntaneously occurring.
3. Human ocular melanoma.

When tissue culture growth was used as the tumor source, $5x10^6$ cells were spun down into a small pellet and resuspended in .1cc of RPMI 1640. Othersolid tumors obtained from elsewhere were processed as follows; all solid tumors were utilized within 48 hours from the time of enucleation. Following removal, the tumor was placed in a sterile petri dish containing RPMI 1640 and pH maintained a 7.4. Using sterile technique, the tumor was excised and minced to about 1 mn^3 which were used as corneal tumor implants.

Tumors used:	Retinoblastoma(RB);	Melanoma(M)
Exp. 2.	Y-79 suspension	Hamster- Greene
Exp. 3.	Human RB	Human and dog melanoma
Exp. 4A.	Y-79 implants	Hamster-Greene grown in rabbit anterior chamber
Exp. 4B.	238-1 implants	Hamster-Greene
Exp. 5.	Y-79 implants	Hamster-Greene
Exp. 6.	Y-79, 238-1 fixed in gluteraldehyde	Hamster-Greene fixed in gluteraldehyde
Exp. 7.	Y-79 implants	Hamster-Greene
Exp. 8.	Y-79 implants	Hamster-Greene

Implantation of tumors

After administering intravenous Nembutol anesthesia(50 per cent) the eye was gently proptosed by pressing on the interior orbital edge with sterile cotton-swab stick and secured in position by securely clamping a fold of skin of the lower lid. With a Bard-Parker

#11 blade, a superficial incision of 1 mm width and .2 mm depth was made in the cornea slightly to one side of the center. Care was taken not to enter the anterior chamber. A malleable iris spatula was introduced into the incision and an oblong pocket formed from the central incision toward a limbla edge. The length of the incision was approximately 3-4 mm, so that the pocket ends 1-4 mm away from the limbal edge. The tumor was placed into the pocket by a syringe when the tissue culture cells were used, and by an iris spatula when solid tumors were used. All prodedures were performed using sterile techniques. No antibiotics were used and no suture is placed.

Observation

The rabbits will be observed daily with a handlight and loupe for any changes in the eyes. Those showing significant changes were photographed with the slit lamp biomicroscope. Clinically, the presence of inflammation was considered to be positive when conjunctival and/or limbal hyperemia and edema with or without exudation were visible. Histologically, an inflammatory infiltrate and location and characteristics of corneal vascularization were noted as they related to the implanted tumor.

Routine histologic sections

The rabbits were sacrificed at intervals following the implantation of the tumors and the eyes were fixed in 10 per cent phosphate buffer formalin for at least 48 hours(63). A corneal meridian appoximately 5 mm in width containing the tumor mass is carefully

removed with a sharp razor blade and placed in alcohol overnight. They were processed in the conventional manner with six micron thick sections being cut and stained with Harris Hematoxylin and eosin for examination under the light microscope(63)

3. Experimental Results

Exp. 1A. The effect of creating the corneal pockets without implantation of any material in two rabbits. On gross examination, these eyes showed minimal reaction. There was mild inflammation mainly characterized by a mild conjuntival and limbal hyperemia in the area adjacent to the corneal pocket. This lasted less than a day, and the eyes became grossly normal in appearance except for a vestige of the incision scar which lasted for 5-7 days. There was no vascular ingrowth and all eyes healed completely within one week without any permanent efidence of inflammatory response resulting from the wound, when observed as long as one month after surgery(Table I). Histologically, the cornea appeared normal without any evidence of inflammation or vascular invasion. The pockets within the stroma healed and the incision scar could not be detected.

Exp. 1B. The effect of the formalin injection into the corneal stroma of one rabbit. 0.1 cc of 10 per cent phosphate buffered formalin was injected into a corneal pocket created in the manner described above. On gross observation, the eyes became severely inflamed in less than 24 hours with marked conjuntival and corneal edema and conjunctival and corneal hyperemia, especially in the area adjacent to the pocket. The eyes continued to show severe

inflammation without any observable corneal neovascularization until the animal died on the 5th day after the injection . The eyes were not available for histologic study.

Exp. 2. The effect of injecting viable tumor cells maintained in tissue culture. Four rabbits were used; their corneas were injected with a prepared suspension of retinoblastoma or melanoma cells. This experiment was fraught with technical difficulties: it was very easy to perforate the cornea during injection of the cells. Also it was difficult to prevent leakage of the injected cells from the corneal pocket due to compression from the corneal stroma. In an attempt to prevent such leakage, the site of corneal incision was sutured in one eye. The pressure applied to the cornea during suturing worsened the leak and thus this technique was discarded. On gross observation, all three corneas containing retinoblastoma cell suspensions developed an inflammatory response, but none was followed by corneal vascularization. Three out of four of the corneas with melanoma cell clumps showed inflammation, and two of them developedcorneal vascularization(Table II). Histologically, a limbal inflammatory cell infiltrate composed of heterophils was accompanied by poorly differentiated capillaries of 7-21 in width with a partial endothelial lining. However, only occasional tumor cells were found on histiological examination, when tumor material used was in the form of cell suspension as in retinoblastoma. This indicates probable loss of tumor cells from leakage.

Exp. 3. The effect of corneal implantation of solid tumors. Twenty-seven rabbits were used. Using spontaneously occurring solid human

and animal tumors avoided the problems associated with cell injection(Table III). Grossly, 2 out of 28 eyes containing retinoblastoma and 11 of 19 eyes containing melanoma showed an inflammatory reaction followed by a corneal vascularization. The inflammatory response occurred within one day following implantation of tumors. The duration of inflammation was approxiamtely 2 days for both tumors; the onset of corneal vascularization was grossly observed approximately 8 days and 5 days following retinoblastoma and melanoma implantation, respectively. After a maximum of 12 days of grossly observable corneal vascular growth, the vessels tended to resolve as noted from their apparent number and their hyperemia. The vessels were no longer visible approximately 20 days after the corneal implantation. Histologically, corneal sections reveal an infiltration of inflammatory cells extending from the limbus toward the tumor implant, with the greatest concentration of cells in the limbal region. Tumors appeared as sequestered foci of clumped cells with pigment release in the center and viable tumor cells in the perephery of the tumor. These inflammatory cells were accompanied by tiny vaxculature of varying sizes(2 RBC's to 7 RBC's) which were sometimes lined by endothelial cells(Fig. 1-2. Fig. 1-5). An eye enucleated 2 days following retinoblastoma implantation showed a histological picture characterized by marked infiltration of heterophils and capillaries from limbus to tumor periphery. The histology of the cornea 7 days and 30 days post tumor implantation shows a similar picture. There appears to be no difference in the characteristics of the vasculature at various periods following the tumor implantation. Also, there appears to be no signigicant difference

in gross as well as histological appearance, between the corneas containing retinoblastoma and melanoma.

Exp. 4. The effect of the corneal implantation of the tumor fixed in formulin. The tumor material was fixed in formalin for 6-24 hours. Some of the tumor was washed in normal saline after formalin fixation (Exp. 4A) while others were not(Exp. 4B). As would be expected, those treated with formalin and implanted without saline-wash produced a much more intense inflammation lasting for a 2-3 times longer period of time(Table IV A). Histologically, there was no difference between the two groups, except for the degree of inflammation and corneal vascularization. However, there was one eye in the A group which showed a diffuse interstitial keratitis which showed a better differentiated vasculature with semblance of an arteriolar wall(rabbit 20LE)(Fig. 1-6).

Exp. 5. The effect of corneal implantation of tumor clumps boiled in normal saline for 20 minutes. This procedure was espected to denature TAF and other protein within the tumor tissue. Unlike the formalin coated tumors, there appears to be no increase either in intensity or duration of an inflammatory response following the tumor implantation. On clinical observation, all boiled tumor implants, both retinoblastoma and melanoma, produced an inflammatory reaction followed by corneal vascularization. The time course of the clinically observable process following tumor implantation was the same as with the viable nontreated solid tumor implantation(Exp. 3). Likewise, the histological appearance was similar: the tumor implants appear as sequestered masses with foci of necrosis, usually in the

center, with release of melanin in melanoma and prominent clumped calcium in the retinoblastoma(Fig. 1-8).

Exp. 6. The effect of corneal implantation of the tumor treated with gluteraldehyde. It has been shown that the skin homografts survived much longer, if treated with gluteraldehyde prior to grafting(80); presumably, gluteraldehyde binds and complexes with the surface antigen and thus reduces the antigenic stimulus and lowers the host immune response to the foreign substance. On gross observation, 3 of 7 eyes showed inflammatory reaction one showed corneal vascularization without any inflammatory reaction (Table 6). On histological examination, however, all corneas show a mild inflammation with few corneas vessels. Compared to the previous ones, there is a moderate decrease in inflammation and corneal neovascularization (Fig. 1-9).

Exp. 7. The effect of intraperitoneal and topical therapy of rabbits with indomethacin prior to and following the corneal implantation of viable tumor clumps. Altogether six rabbits were used. 25 mg. of powdered indomethacin was dissolved in 2 cc of phosphated buffer solution pH 8.4-8.8 and given intraperitoneally twice a day for two days prior and two days following the tumor implantation. For topical indomethacin treatment, a 10 per cent fresh solution was prepared so that each solution was not used for longer than 30 hours. 2-3 drops of topical indomethacin solution were placed in each eye daily and observations recorded. On gross observation, none of the three corneas containing live tumor cell suspensions showed extensive inflammation or vascularization. Of the seven

corneas containing the boiled tumor implants, six did not show any gross evidence of inflammation; two of the seven corneas were vascularized (Table 7). It is important to note that there was no difference between the viable and boiled tumor implants in inducing corneal vascularization in the animals treated with indomethacin.

Histologically, the cornea appears similar to the previous ones, however, the degree of inflammation and corneal vascularization was reduced as with gluteraldehyde treatment. However, the tumor cells are scattered about the stroma without any localization, since the cell suspension rather than clumps was used as the tumor source.

Exp. 8. The effects of tumor implantation in rabbits treated with 900-1100R. Six rabbits were radiated to reduce the host inflammatory reaction. The immune suppressing dose of radiation was calculated by the nuclear physicist and the radiation equipment used for human radiotherapy was utilized. The radiation technologist administrated the actual radiation prodedure on the rabbits which were contained in a tight wooden box. A total body radiation was accomplished without a direct exposure to the eye. On clinical observation, 3 of 12 showed inflammatory reaction with only one going on to show faint corneal vascularization. Histologically, there was a marked reduction of corneal inflammation or vascularization(Table 8); of 12 corneas, 2 retinoblastomas and 2 melanomas showed evidence of few small capillaries adjacent to the tumor mass. In 8 of them, there was almost negligible inflammatory cells and vascularization. The difference between the irradiated and

nonradiated hosts were striking, given the same tumor implantation (Fig. 1-10. Fig. 1-11)

4. Discussion and conclusion.

The mechanism of corneal vascularizaiton has been a subject of much debate. Studies on standardized burns in rabbit cornea show that corneal neovascularization involves a diffusable factor which may be histamine or other chemotactic substances. However, local hypoxia or accumulation of acid metabolites after corneal injury may in themselves be responsible for vascularization (64).

In conditions such as Eale's disease and diabetic retinopathy in which ischemia arises as a result of vascular occlusion, the responsible agent is said to be anoxia, which acts by releasing vascular growth promoting factors (10).

A decrease of compactness of corneal tissue may also be responsible for initiating the vascular growth(59, 67). A state of suboxidation, and thus a stimulus for neovascularization, was always present within the corneal stroma. This stimulus could only exert its influence when vessels had the possibility of advancing as a result of corneal edema and loosening up of the normally tight stroma. However, there is no evidence that a reduction of tissue compactness by chronic edema, even of long duration, has any influence on new vessel formation outside the cornea(63).

It has been observed that there is no apparent need for a vascular supply to allow the continued growth of the tumor and to establish anatomic contiguity of the tumor and host tissue(33,41, 48, 91).

Gimbrone, Folkman, and coworkers proposed a specific relationship between the tumor and the host in initiating the endoproliferative response(39, 41, 95).

Although it is conceptually more appealing to invoke the presence of some special factor to explain the tumor associated vascularization, the present study would call for a closer examination of the existence and role of TAF. One may ask the following questions:

1. If TAF exists, does it act directly on the host vascular source or via some other mechanism?
2. Supposing that this blood vessel inducing factor exists, is it tumor elaborated substance or is it something unrelated to neoplasm? Is the corneal vascularization a result of a balance of inflammatory and neoplastic effects?

The suggestion that there is some communication between the tumor and host vascular source has been made by numerous other studies(37, 46, 89). However, the nature of this tumor elaborated protein substance and its mechanism of action are still unknown. It is unknown whether TAF acts by inducing direct vascular proliferation or by removing an inhibition that normally prevents the unrestrained vascular growth or by promoting some cellular surface interactions or by causing inflammations. Furthermore, it has not been adequately shown how one can distinguish the neovascular response secondary to TAF from that secondary to other factors such as inflammatory substances, eg. the vasoactive amines.

In this study, the fact that a majority of corneal neovascularization was preceded by a period of inflammation suggests that the mechanism and effects of inflammation have at least some role

for corneal neovascularization. It is not clear whether this is manifested through TAF or some other factors such as the action of the tumor as a foreign body, an effect of wound healing, and/or an immune response that produces capillary proliferation with the inflammation as a parallel process.

The preceding experiments and results would indicate that inflammation and/or immune response to the foreign body implantation have at least some role in producing corneal neovascularization following tumor implantation, and that the role of TAF in corneal vascularization is inconclusive.

It has been shown that live tumor cells in the corneal pockets produced local interstitial keratitis. The degree of neovascular response seemed to be directly correlated with the degree of inflammation. It is impossible by conventional light microscopy to distinguish the capillaries seen in the midst of inflammatory cells from those seen among the tumors cells.

It cannot be shown that the corneal neovascularization following viable tumor implantation (Exp. 3) was induced by TAF. Various measures were taken to elucidate the neovascularization occurring in the absence of any TAF influence, so that the effect of inflammation and TAF may be observed independently. It seems reasonable to expect a lower incidence of corneal vascularization by a devitalized tumor material, particularly following boiling or formalin fixation, but the histologic picture is identical to that seen with viable solid tumors implantation. The only difference was markedly greater degree of inflammatory response when tumor implants were

used without saline washing. Without knowing which corneas came from which experiment, one would be unable to distinguish the formalin fixed implants from a viable nontreated tumor implant except for the degree of inflammation. Similarly, all the boiled tumor material implanted in the cornea produced neovascularization. This would suggest that whatever the effect of TAF, implantation of nonviable tumors with denatured protein within the cornea acts as a sufficient foreign material, producing ocular inflammation and subsequent corneal vascularization.

If it is the inflammation rather than or in addition to the specific TAFthat produces the corneal vascularization, the interference with the host's response or a reduction of antigenic stimulus should show a considerable decrease in the degree of inflammatory response as well as in the corneal vascularization. The gluteraldehyde treatment of the tumor implants appeared to reduce the degree of inflammation and corneal vascularization(Table 4). It is possible that although the tumor as a foreign body caused local inflammatory response, gluteraldehyde may have prevented the antigenic stimulation by binding to the tumor surface antigens. However, the immunological role of gluteraldehyde is not established. Treatment with indomethacin also seemed to reduce the inflammatory reaction and vascularization. The most striking difference was noted with radiation treatment of the host proir to tumor implantation; there was a remarkable decrease in inflammation as well as corneal neovascularization. The induction of corneal vascularization following tumor implantation within the corneal stroma appears to a large extent to be mediated by a process of inflammation.

In addition, the hypothesis that retinoblastoma may lack or has insufficiency of TAF and that thus has a role in spontaneous regression

of the tumor cannot be substantiated. There was questionable difference between retinoblastoma and melanoma in response to tumor implantation in the cornea, and there was no significant delay in vascular growth into the cornea between these two tumors.

It is probable that the corneal model as used here, a slight modification of Folkman's procedure, may be a poor one for TAF(39). It is possible that the compactness of the corneal stroma may produce pressure and degenerative changes in the tumor cells and cause inflammation and its resultant vessel formation before TAF ever has a chance to exert its effect.

V. The role of calcium in spontaneous regression of retinoblastoma; an experimental study.

1. Introduction.

The presence of calcified foci within the area of necrotic retinoblastomas has long been observed and documented and has led both clinicians and investigators to suspect an etiological role of calcium in its spontaneous regression. This study proposes to determine whether retimoblastoma cells are particularly susceptible to a high concentration of calcium when compared with other tumors. one can study this by placing tissue culture cells of various tumor lines in the media which contain various concentrations of calcium. Parallel studies will use media without extra calcium and also by using the media containing the molar-equivalent amount of extra NaCl in order to control osmolarity effect.

2. Methods and Materials.

Procurement of tissue culture cells:

Human Retinoblastoma (Y-79)----------from Dr. D. Albert's Laboratory

Human Neuroblastoma(88)----------from Dr. D. Albert's Laboratory

Hamster-Greene Melanoma(22)----------from Dr. D. Albert's Laboratory

Burkitt's Lymphoma(RAJ)----------from Dr. G. Miller's Laboratory

Lymphoblastic Leukemia(4265,SKL-7)----from Dr. J. Bertino's Laboratory

Human Fibroblasts(WI-38)----------from Dr. G. Hsiung's Laboratory

Procedure:

RPMI 1640 was used as a medium to prepare the suspension cultures of different cell lines. Calcium is present in medium

1640 as $Ca(NO_3)_2$ $4H_2O$ in concentration of 0.42 millimolar. In order to increase the calcium by ten times, 4.2 millimoles of $CaCl_2$ was used by calculating its molecular weight (110) and the amount to add by multiplying it by 4.2. Thus 231 mg. was dissolved in 500 cc of medium, but because of precipitation of calcium salts, the actual dose of calcium was greatly lowered. In order to obtain the final prepared media containing the desired tenfold concentration, 23 mg. of $CaCl_2$ was dissolved in 50 cc of RPMI 1640. The final concentration of calcium was to be approximately 46 mg%, as checked by the clinical chemistry laboratory. In a control group, a molar-equivalent amount of NaCl was added to RPMI 1640 instead of $CaCl_2$.

Into each of nine sterile falcon flasks, the appropriate media and $1\text{-}5X10^6$ tumor cells were placed. All cultures were incubated in 37° C. Each flask was replaced by a fresh medium every three days to insure an adequate nutrition and to remove the waste material from the environment.

Experimental Study:

(I) First, a preliminary experiment was done using three cell lines, human retinoblastoma, neuroblastoma, and hamster melanoma to 1) find the duration of exposure to the media containing ten-fold concentration of calcium that was sufficient to damage the cells irreversibly, and 2) to simultaneously control the effects of osmolarity using the media containing the molar-equivalent amount of the extra sodium.

(II) Second, each cell line was exposed to the increased concentration

concentration of calcium for varying periods fom one half the doubling time to several times the doubling time; for human lymphoblastic leukemia, 4265 and SKL-7, whose doubling time was approximately two days, cells were exposed for 6 hours, 17 hours, and four days; for all the other cell lines whose doubling time was approximately two days, cells were exposed one, two, and three days. At the end of each exposure period, cells were placed in the normal media. Lymphoblastic Leukemia cell lines (4265, SKL-7) were placed in their respective normal media; RPMI 1640 with 15% bovine serum albumin for 4265, and Fischer's media with 10% horse serum for SKL-7(Dr. Bertino's Laboratory). All other cell lines were placed in the media RPMI 1640.

I) Preliminary Study: Calcium Concentration in the media for B) and C) of all groups was 49 mg.%.

Group I: A) Retinoblastoma in medium 1640
B) " in medium with increased sodium
C) " in medium with increased calcium

Group II: A) Melanoma in medium 1640
B) " in medium containing increased sodium
C) " in medium containing increased calcium

Group III: A) Neuroblastoma in dedium 1640
B) " in medium containing increased sodium
C) " in medium containing increased calcium

II) Sensitivity of various neoplastic and a noneoplastic cell lines to an increased concentration of calcium by ten-fold in the environment. Measured calcium concentration was 50 mg.%.

Tumor Type	Period of exposure to increased calcium concentration			
	Group 1	Group 2	Group 3	Group 4
A) Retinoblastoma	1 day	2 days	3 days	4 days
B) Neuroblastoma	"	"	"	"
C) Burkitt's RAJ	"	"	"	"
D) Melanoma (Hamster-Greene)	"	"	"	"
E) WI-38	"	"	"	"
F) Lymphoblastic Leukemia				
4265	6 hrs.	17 hrs.	4 days	
SKL-7	"	"	"	

After a given peroid of exposure to the increased calcium concentration, cells were returned to their own respective normal media.

Observation:

The histopathologic study of the cellsin the culture was carried out in several ways:

a) Cells were examined daily both grossly and microscopically and micrographs were taken as they grew in vitro.

b) Smears were made of all cell lines whenever significant changes occurred.

c) Tissue culture material was fixed in Zenker formalin solution and sections stained with Hematoxylin and Eosin. Calcium staining was done when there were significant significant cellular calcification.

d) Specimens from the tissue culture system were studied under the electron microscope. Tumor material was centrefuged into a pellet and minced into fine pieces. Fixation of tumors and pellet was carried out in either Dalton's chrome osmium fixative for one hour or three per cent phosphate-buffered glutaraldehyde-sucrose for one hour followed by chrome osmium fixation. Tissue was dehydrated in graded ethyl alcohol and embedded in Epon-Araldite mixture. Ultrathin sections were cut with an LKB microtome and double stained in uranyl acetate followed by lead citrate. Micrographs were taken with a Siemens I electron microscope using a 80 kv. accelerating voltage and a 50u objective aperture.

3. Results of the calcium sensitivity study:

The preliminary study using three cell lines showed that in human neoplastic cell lines, four-day exposure to increased calcium concentration could effectively destroy the cells irreversibly. The study also showed that a sodium increase by the molar equivalent amount did not effect the cells. Therefore, one can say that the damage done to the cells placed in the increased calcium environment may ha e been due to factors other than the increased osmolarity exerted by the additional ions.

Retinoblastoma: The control retinoblastoma cells in the normal media showed clumps of round cells freely floating. After two days of exposure to the higher calcium concentration, microscopic

examination revealed a grossly undisrupted cell integrity. However, the cells were no longer in large clumps as in the control; cells were more scattered and their cytoplasm had a granular appearance(Fig. 2-1, Fig. 2-2). After a four day exposure, more cells appeared smaller in size and more variable in shape, and occurred as separate single cells, and showed fine granularity of cytoplasm and intercellular stippling that probably reflected calcium precipitates.

Histologically, after a one day exposure to calcium one could see some disruption of epithelial integrity and loss of cellular contents. After a three day exposure, many cells showed pyknosis, karryorrhexis, and broken epithelium with pink amorphous material intercellularly and possibly intracellularly as well.

Calcium staining showed scattered extracellular and possibly intracellular precipitates. The calcium precipitates resembled crystals or darkly staining beads concatenating especially around the cell border and within the cellular debris. Electronmicroscopic examination revealed that the calcium was mostly in the extracellular region(Fig. 2-7). Intracellular calcium was observed only when ther was a visible violation of the cell membrane.

Replacement by the normal media after each successive exposure to calcium did not restore th cells to any considerable degree when the cells were exposed for longer than 2 days. In the preliminary study, four-day exposure failed to regenerate

the destroyed cells. However, some cells showed a loss of membrane integrity as early as after a one-day exposure, and these did not regenerate even when returned to the normal media for five days. They appeared as darker, smaller and degenerated cells in the background among other healthier floating cells.

Neuroblastoma:Cells seemed to be as vulnerable as the retinoblastoma cells to the increased concentration of calcium. The control cells in the normal media grew as a sheet of cells. Cells exposed to calcium for two days showed the microscopic appearance of increased clumping with blurriness of the cell borders. Like the retinoblastoma, these cells underwent irreversible damage as shown by the absence of signs of improved viability of cells after five days in the normal medium(Fig. 2-2).Histologically, one day exposure caused granular changes of the cytoplasm and disruption of membrane integrity. Three-day exposure caused tearing of the cells with spiculated cell borders, loss of cellular contents, and shrunken size. A smear preparation showed a scattered cellular debris resembling remnants of degenerated cells and their fragments with calcium precipitates within them as small round dark-staining dots. Calcium was also seen independently as amorphous crystals.

Burkitt's lymphoma cells: like retinoblastoma and neuroblastoma, they showed similar changes after two days of exposure to the increased calcium concentration. Also, histologically, the same membrane disruption was observed after one day exposure. Three day exposure showed histological changes unlike the previous

two; cells resembled target cells with darker staining in the center and the periphery and a paler region in between. The darker material on closer view showed a beady appearance which may have been calcium deposits. Cells showed some regeneration but damaged cells even as early as at one day following exposure remained without any change toward regeneration after five days in the normal media (Fig. 2-4). A four-day exposure gave a histological appearance of a scarcity of cells and a large number of calcium crystals resembling the tightly packed spokes of a wheel.

Lymphoblastic leukemia cells, 4267 and SKL-7: These cells have a much shorter doubling time. Therefore, an equivalent duration of exposure to the increased calcium as the other cells was achieved by exposing them to the corresponding turnover periods. One day in retinoblastoma would correspond to six hours in lymphoblastic leikemia, etc. Microscopic changes in these cell lines were not observable after a 22 hour exposure but histological examination showed changes as early as after a six hour exposure in both cell lines. After a six hour exposure, the histological examination revealed membrane disruption, nuclear fragmentation and extracellular calcium deposits. At 22 hour exposure, disruption of cell integrity with fragmentation and the release of cellular elements were more common. Furthermore, there was a ballooning of cells with a bubble-like appearance of cytoplasm. There was no regeneration of cells already damaged when returned to the normal concentration of calcium. Preparations stained for calcium

showed scattered calcium deposits mostly in the extracellular space and over the cellular debris(Fig. 2-5, Fig. 2-6).

Melanoma:While the human tumor cells' were vulnerable to a higher calcium level in their enrironment, the animal tumor cells tested showed resistance and resiliency toward such ionic changes in the environment. Cytoplasmic granularity and epithelial disruption, seen early in other cells, were not observed until approximately the fourth day of exposure to the higher calcium concentration(Fig. 2-3). Histological examination showed an aggregation of cells with blurring of cell borders and scattered calcium deposits.A four-day Exposure showed membrane disruption with cellular fragmentation and degeneration . However, the melanoma cells were distinct from the other cell lines in that the regeneration occured promptly within one day when returned to the normal media even after the cells have been exposed to the higher concentration of calcium for as long a four days.

Human Fibroblasts (WS-38): Unlike malignant cells in general, susceptibility to such a change proved devastating to a line of normal human cells, human fibroblasts, WI-38. Both microscopically and histologically there was a prompt loss of fibrils and shrinkage of cells to small round cells of irregular size with spiculed borders. Cells were destroyed most promptly and were not restored after being returned to the normal media.

4. Discussion and Conclusion.

Increased levels of calcium in this study accelerated the cellular degeneration of all tumor cell lines equally, except for melanoma. Retinoblastoma cells were not particularly more vulnerable to calcium exposure with respect to either degree of ionic concentration or the duration of exposure. Non-tumor cells such as human fibroblasts, however, were destroyed with greater susceptibility.

The calcium deposition within the eye may be a natural consequence of cellular degeneration, although the eye is affected less frequently than otherorgans such as lungs, kidney, and gallbladder(104). The pathogenesis of calcification in the eye or in other soft tissue was believed by some to be a "frequent end-change in degenerating tissues which are accessible to body fluids, the change being probably a physical one, depending on a low carbon dioxide tension due to metabolic inactivity" (104). This is supported by the fact that calcification is seen almost invariably in the necrotic foci of tumors usually farthest remove from the vascular supply.

However, other degenerative changes in the eye do not lead to calcium deposition, notably sympathetic ophthalmia and and melanoma. Samuels reviewed 100 globes remove because of sympathetic ophthalmia and 84 globes because of melanosarcoma but did not find any eye with calcium deposition(104). One may wonder whether it is a particular type of degeneration such as

hypoxia that leads to calcification. Whatever the cause of calcification in retinoblastoma may be, it is evident that retinoblastoma cells were not more vulnerable to higher concentrations of calcium in the environment as compared to other cell lines. Therefore, the hypothesis that retinoblastoma cells may be more susceptible to calcium and that calcium may have an etiologic role in the spontaneous regression of retinoblastoma cannot be supported.

VI.TABLES AND FIGURES.

TABLE I. Exp. 1A.

The effect of creating corneal pockets alone without any tumor implantation.

Rabbit Id. #	presence of conjuntival or limbal inglammation	duration of grossly observable inflammation	onset of corneal vascularization
0 RE	+	less than 24 hours	-
0 LE	+	less than 24 hours	-
1 RE	+	less than 24 hours	-
1 LE	+	less than 24 hours	-

TABLE II. Exp. 2.

The corneal implantation of viable tumor cell suspension.

Rabbit Id #	tumor source	Presence(+) or absence(-) of ocular inflammation	Duration of inflammation, as grossly observed	Onset of inflammation from the day of tumor impl.
2 RE	RB(Y-79)	+	1-4	-
4RE	"	+	1-5	-
33RE	"	+	1-2	-
2LE	M(22)	+	1-3	1
3LE	"	-	-	-
4LE	"	+	2-9	3

RB: Retinoblastoma

M: Melanoma

RE: right eye

LE: left eye

TABLE III. Exp. 3.

The effect of corneal implantation of viable retinoblastoma and melanoma cell mass derived from the solid human and animal tumors.

Rabbit Id. #	Tumor source	Ocular inflammation	Duration of grossly observable inflammation	Onset of corneal vascularization	
7RE	RB(human)	+	6-7	7	
8RE	"	-	-	-	
9RE	"	+	6-7	9	
11RE	"	+	3-4	5	
12RE	"	+	2-3	5	
13RE	"	-	-	5	
14RE	"	+	2-3	2	
15RE	"	+	8-9	10	
16RE	"	-	-	-	
17RE	"	+	3-4	8	
27RE	RB(238-1)	+	1-2	1	
28LE	"	+	2-4	7	
29LE	"	+	1-6	7	
30LE	"	+	1-4	4	
31LE	"	-	-	-	
32LE	"	+	1-4	4	
55RE	RB(Y-79)	+	-	-	
59RE	"	-	-	-	
65RE	"	+	1-2	3	

CON'T

Con't. from Table III. Exp. 3

Rabbit Id. #	Tumor Source	Ocular Inflammation	Duration of grossly observable infla.	Onset of corneal vascularization
7LE	M(human)	+	1-4	2
8LE	"	+	1-5	2
9LE	"	(tumor fell out)		
11LE	M(dog)	-	-	-
12LE	"	+	2-6	6
13LE	"	+	2-3	10
14LE	"	+	1-3	4
15LE	"	+	2-3	5
16LE	"	+	1-2	-
17LE	"	+	1-3	3
27LE	M(Hamster-Greene)	+	1-2	-
28RE	"	+	1-2	-
29RE	"	+	4-6	7
30RE	"	-	-	-
31RE	"	-	-	-
32RE	"	+	1-3	4
47LE	"	+	4-5	-
48LE	"	-	-	-
49LE	"	-	-	-

TABLE 1V. Exp. 4A and 4B

The effect of corneal implantation of tumor mass treat with formalin without washing in saline(A) and with washing in saline(B).

	Rabbit Id. #	Tumor Source	Ocular infla mmation	Duration of grossly observable infla.	Onset of corneal vascularization
4A)	19RE	RB(Y-79)	+	1-6	5
	20RE	"	+	1-6	5
	21RE	"	+	1-5	-
	22RE	"	+	1-10	5
	19LE	M(Hamster-Greene)	+	1-5	5
	20LE	"	+	1-5	6
	21LE	"	+	1-5	6
	22LE	"	+	1-7	5
4B)	24RE	RB(238-1)	+	1-2	4
	25RE	"	+	1-2	4
	26RE	"	+	1-3	4
	34LE	"	+	1-2	-
	55LE	"	+	1-2	-
	56LE	"	+	1-2	-
	57LE	"	-	1-2	-
	60LE	"	+	1-2	-
	67RE	"	+	1-2	3
	24LE	M(Hamster-Greene)	+	1-2	4
	25LE	"	+	1-4	2
	26LE	"	-	-	-

TABLE V Experiment 5.

The effect of corneal implantation of tumor boiled in saline for 20 minutes.

Rabbit Id. #	Tumor Source	Conjunctival Inflammation	Duration of grossly obs. inflammation	Onset of corneal neovascularization
50RE	RB(Y-79)	+	1-3	5
51RE	"	+	1-3	4
56RE	"	+	1-3	4
57RE	"	+	1-2	2
50LE	M(Hamster-Greene)	+	1-3	5
51LE	"	+	1-3	5

TABLE VI. Exp. 6

The effect of corneal implantation of tumor mass that have been fixed in gluteraldehyde.

58LE	RB(Y-79) without saline wash	+	1-2	-
59LE	"	+	1-5	died on 5th day
61LE	" without saline wash	+	1-6	-
67LE		-	-	-

TABLE VII. Exp. 7.

The effect of intraperitoneal and topical threrapy of rabbits with indomethacin prior and following the implantation of viable tumor mass or cell suspension.

Rabbit Id. #	Tumor source	Ocular inflammation	Duration of observable infla.	Onset of corneal vascularization
33RE	RB(Y-79) mass	+	1-2	-
36RE	"	-	-	-
35LE	"	-	-	-
33LE	M(Hamster-Greene)	+	1-2	-
35RE	RB(Y-79) suspension	-	-	-
40RE	"	-	-	-
38RE	M(Hamster-Greene)	+	1-5	6
39LE	"	-	-	-
40LE	"	-	-	4

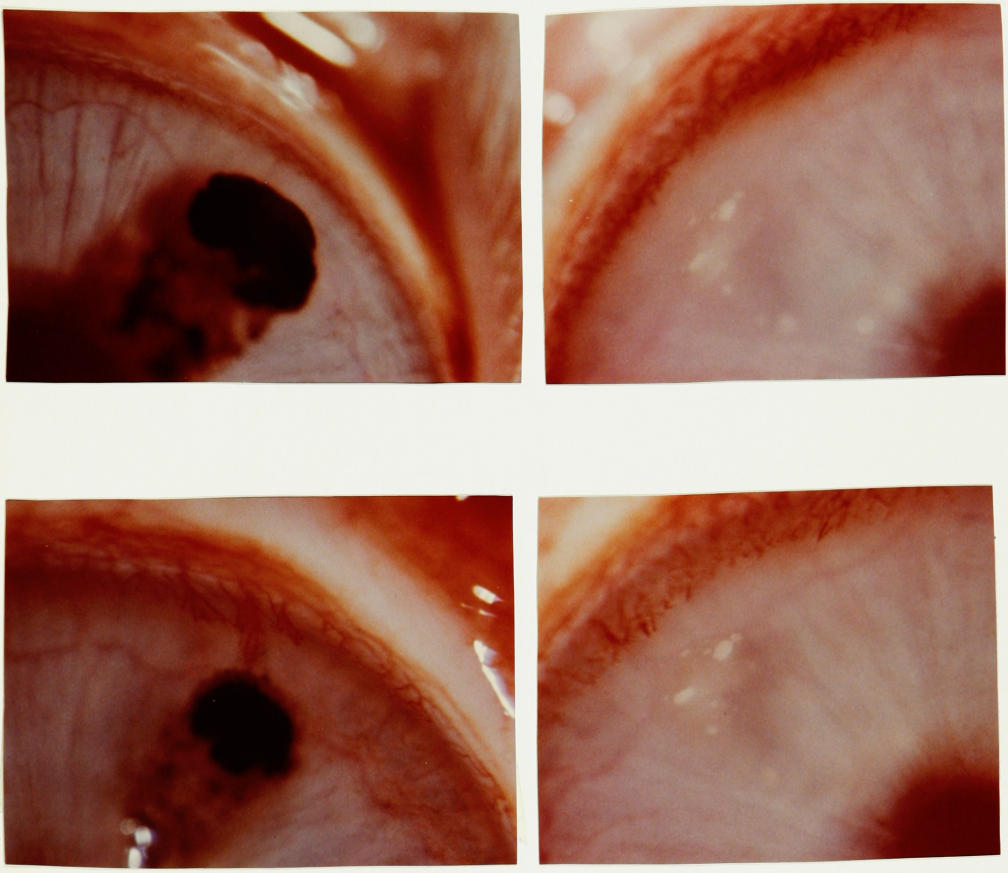
TABLE VIII. Exp. 8

The effect of corneal implantation of viable tumor mass in rabbits treated with an immune depressing dose of radiation (900R)

Rabbit Id. #	Tumor Source	Ocular inflammation	Duration of obs. inflammation	Onset of corneal vascularization
41RE	RB(Y-79)	-	-	-
42RE	"	+	2-3	5
43RE	"	-	-	-
44RE	"	-	-	-
45RE	"	-	-	-
41LE	M(Hamster-Greene)	-	-	-
42LE	"	-	-	-
43LE	"	-	-	-
44LE	"	-	-	-
45LE	"	-	-	-
53RE	RB(Y-79)	+	1-2	-
53LE	Rb(Y-79) formalin fixed	+	1-2	-

Fig. 1-1

Slit-lamp stereomicroscope photographs showing the vascular growth following tumor implantation: melanoma (1) and retinoblastoma (2)



(1) Melanoma

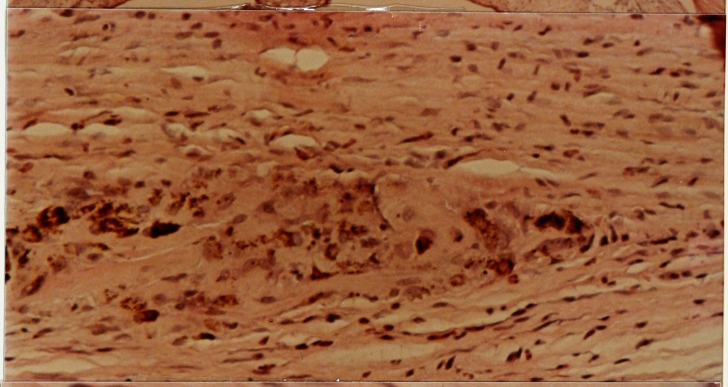
(2) Retinoblastoma

Fig. 1-2

The histological picture of rabbit cornea obtained 17 days after the corneal implantation of viable melanoma.

17 LE viable human retinoblastoma (10x magnification)

Periphery of the tumor mass (65x magnification)



Vessels adjacent to the tumor region (160x magnification)

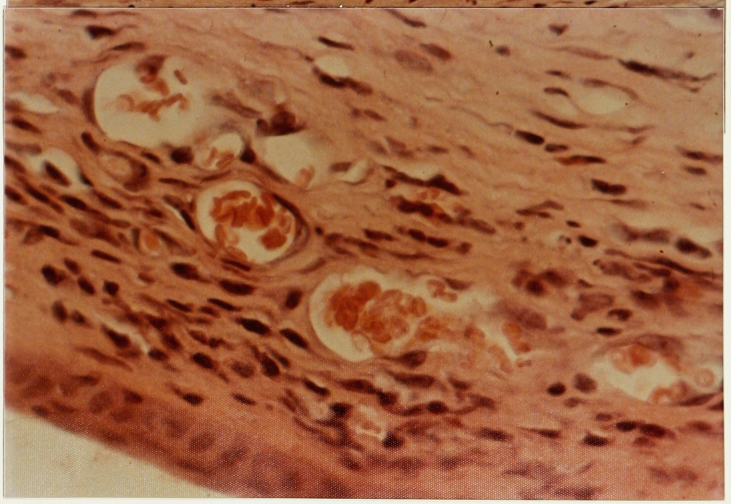
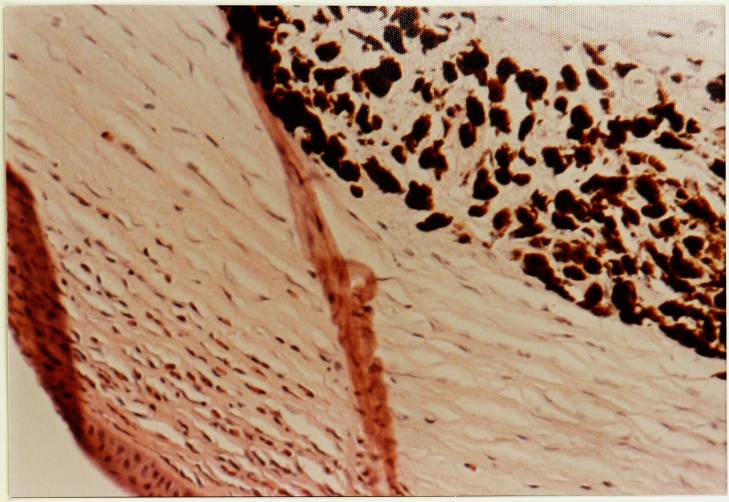
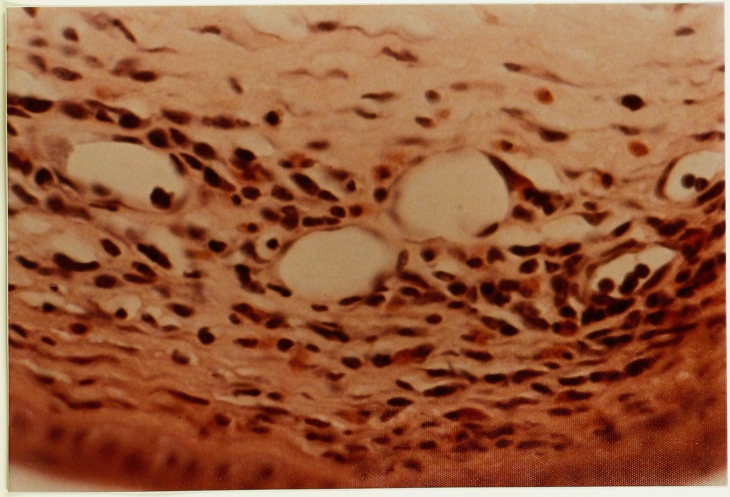


Fig. 1-3

The histological picture of rabbit cornea obtained 9 days after the corneal implantation of viable melanoma.



14 LE * 65



* 160

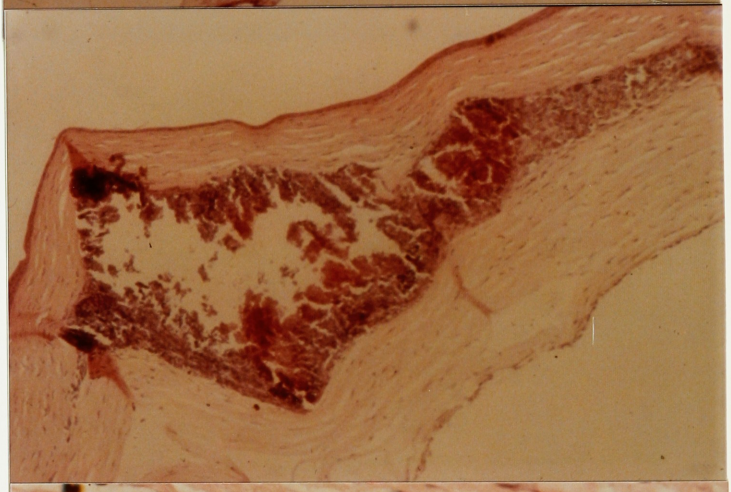
Vessels near the tumor

Fig. 1-4

The histological picture of the cornea obtained 9 days after the corneal implantation of human retinoblastoma.

Magnification
*10

* 25



* 160

Vessels near the tumor

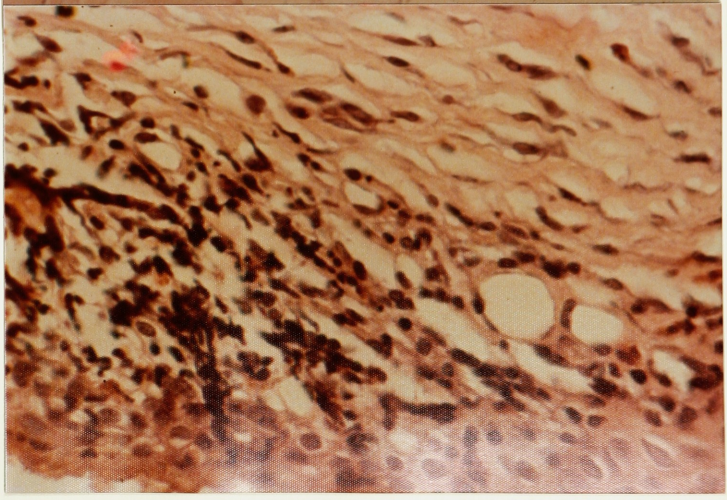
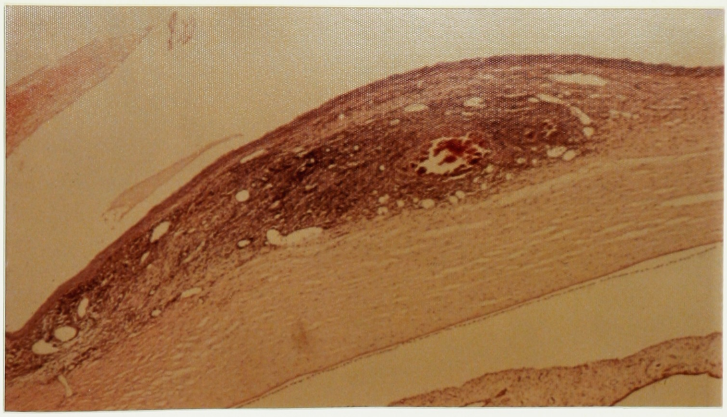


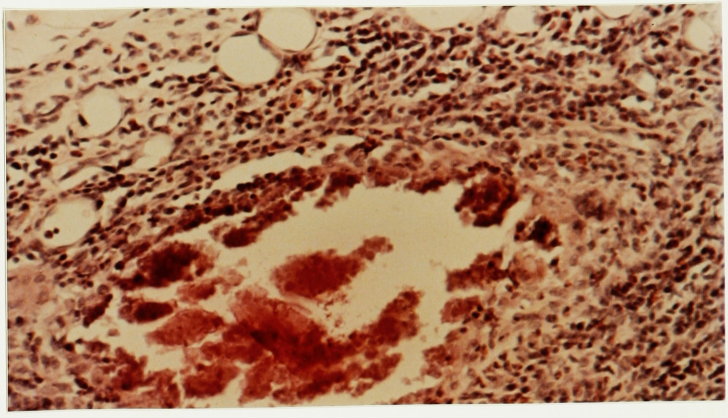
Fig. 1-5

The histological picture of rabbit cornea obtained 13 days after the corneal implantation of viable human retinoblastoma

13 RE ×25



13× 65



×160

vasculature near the tumor

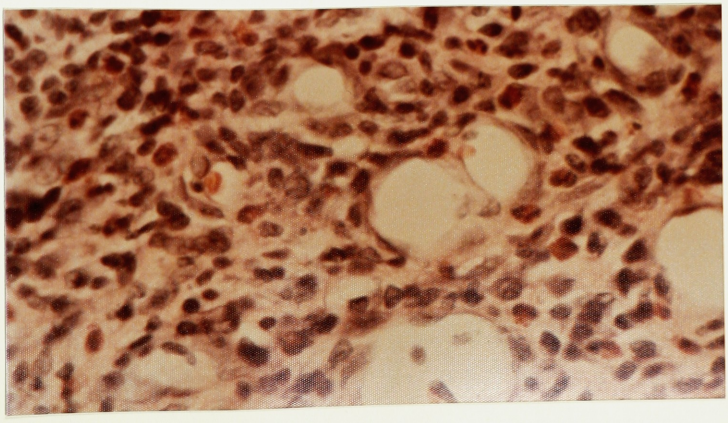
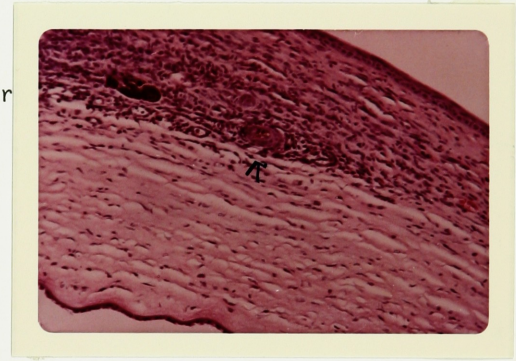


Fig. 1-6

The histological picture of rabbit cornea obtained 9 days after the corneal implantation of melanoma fixed in formalin without washing in saline.

20LE *40

*100 shows the area of the tumor marker: melanin pigment) with severe inflammation: note few well differentiated vascular endothelium(arrow)



*400 shows a closer view of tumor cells with pigment degeneration with heterophils and capillaries.

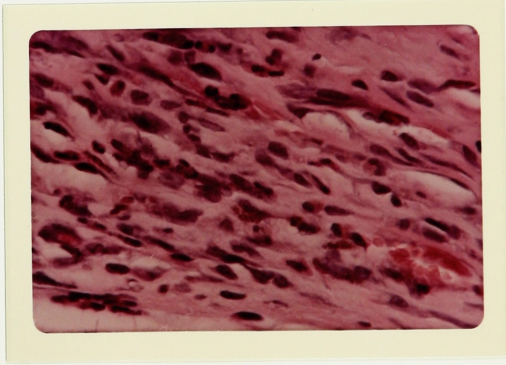
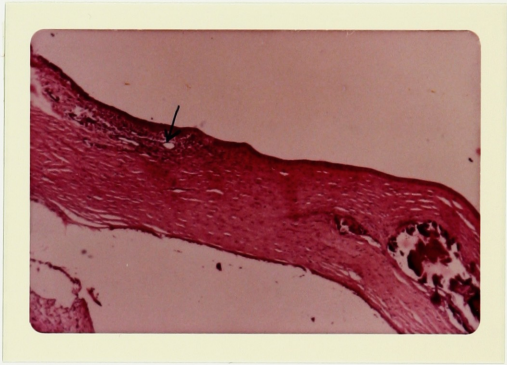


Fig. 1-7

The histological picture of rabbit corneal obtained 2 days after the corneal implantation of retinoblastoma fixed in formalin with saline washing.

3lLE *40 shows
the sequestered tumor mass
with limbal inflammation:
note the formation of capillaries among the
inflammatory cells(arrow)



*400 shows the magnification
of the limbal area with the sinusoid-
like capillary formation(arrow)

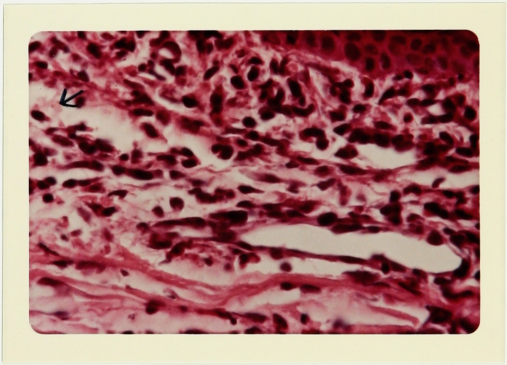
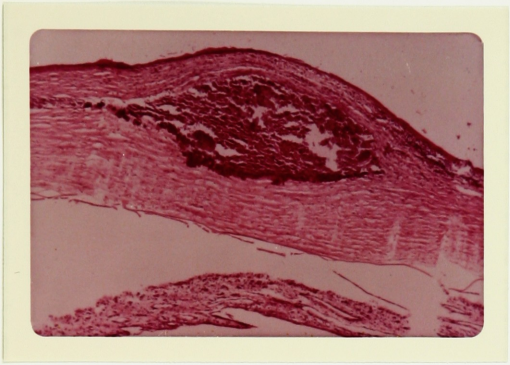
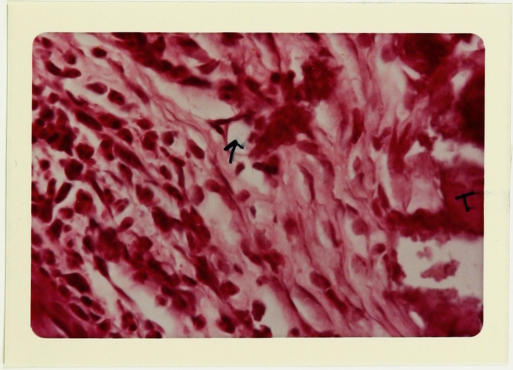


Fig. 1-8

The histological picture of rabbit cornea obtained 5 days after the corneal implantation of retinoblastoma boiled in saline.



57 RE * 40



57 RE * 400

Note scattered heterophils and rudimentary capillaries near the tumor mass (arrow)

Fig. 1-9

The histological picture of rabbit cornea obtained 8 days after the corneal implantation of retinoblastoma fixed in gluteraldehyde and washed in saline.

58LE * 40

58 LE * 400
Near the
tumor mass.

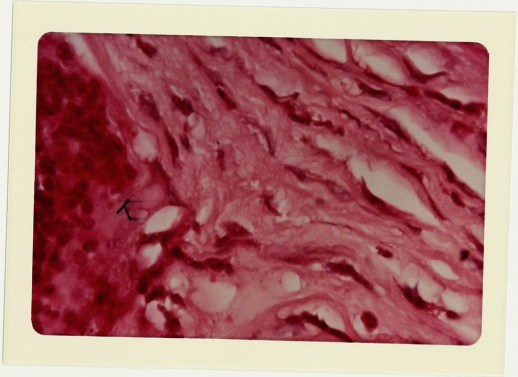
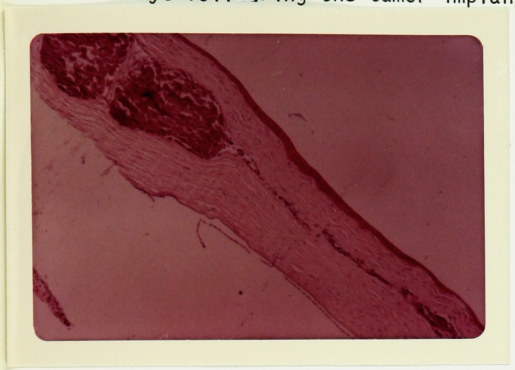
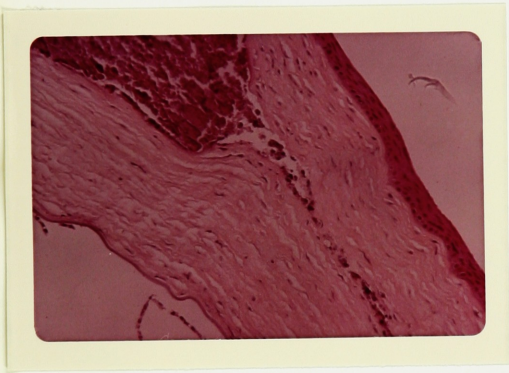


Fig. 1-10

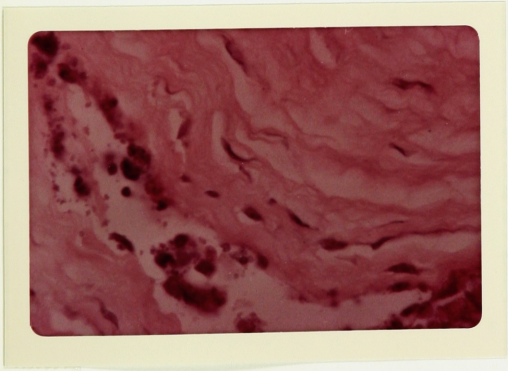
The histological picture of the cornea with retinoblastoma(viable) implanted inr rabbits that have been radiated with 950 R, sacrificed 7 days following the tumor implantation.



43 * 10



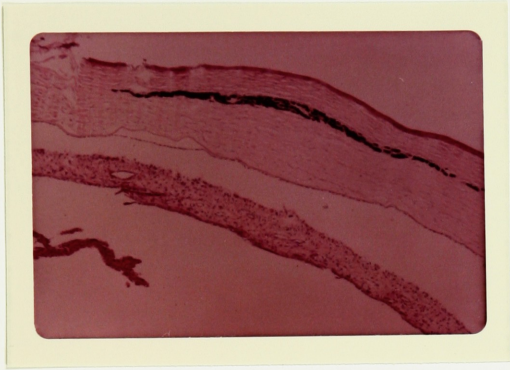
43RE * 100



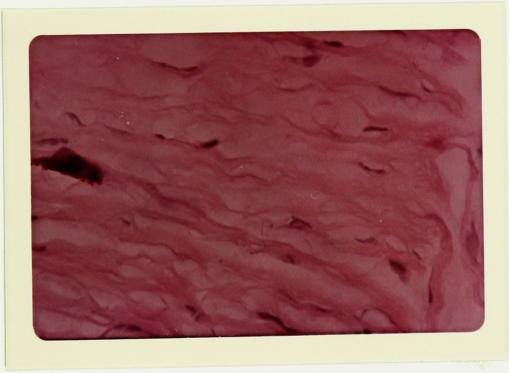
43 RE * 400
Note lack of inflammation and Neovascularization

Fig. 1-11

The histological picture of the cornea with melanoma(viable) implanted in rabbits that have been radiated with 950 R, sacrificed 7 days following the tumor implantation.



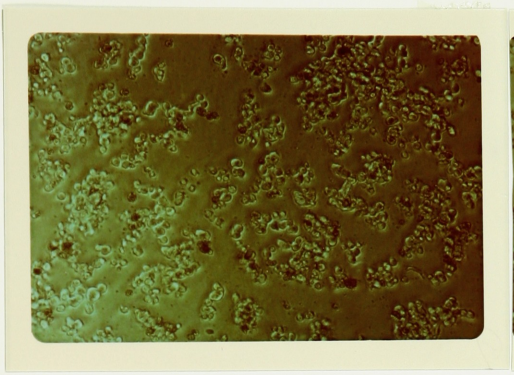
43 LE * 40



43 LE * 400

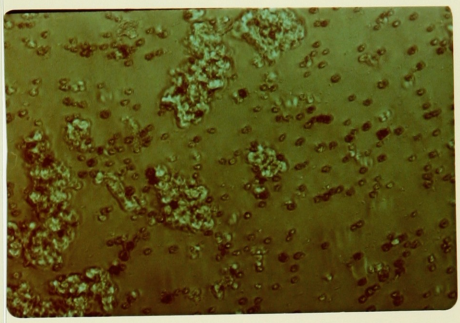
Fig. 2-1

Retinoblastoma cells after varying time periods of calcium exposure .

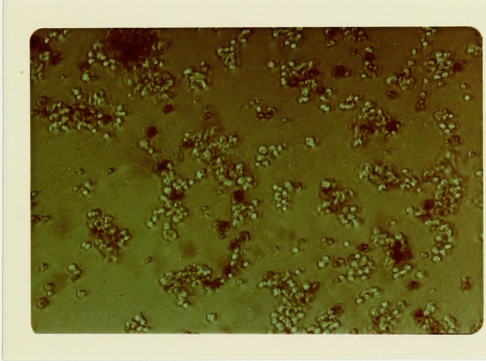


1 day exposure to increased concentration of calcium by ten-fold

followed by

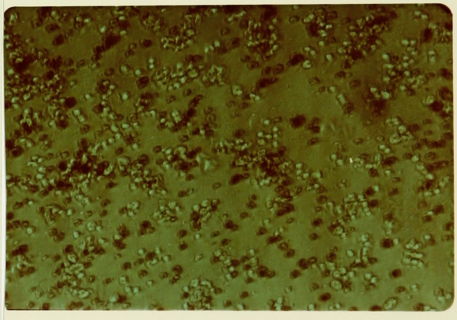


4 days of reimmersion in the normal media

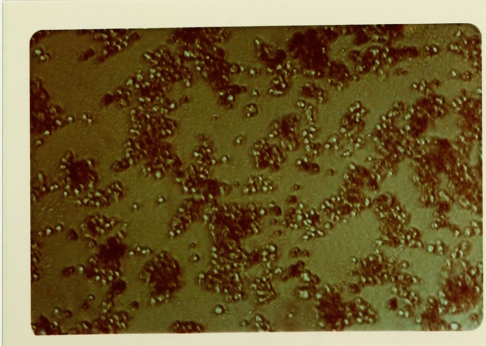


2-day exposure to calcium

followed by



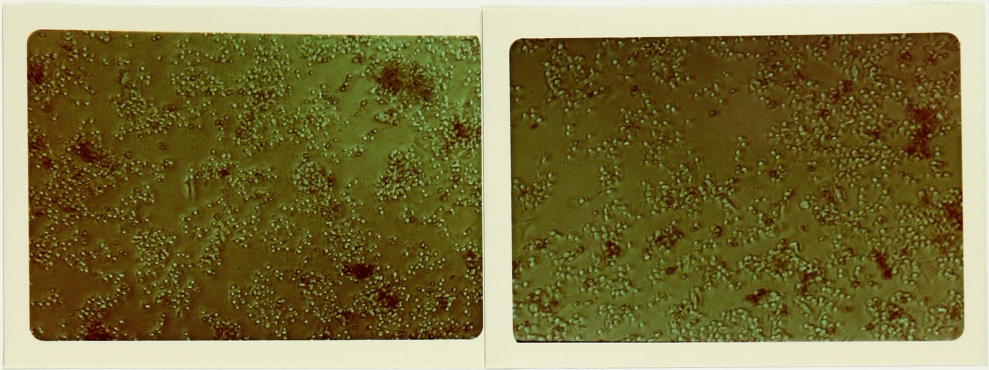
5 days of reimmersion in the normal media



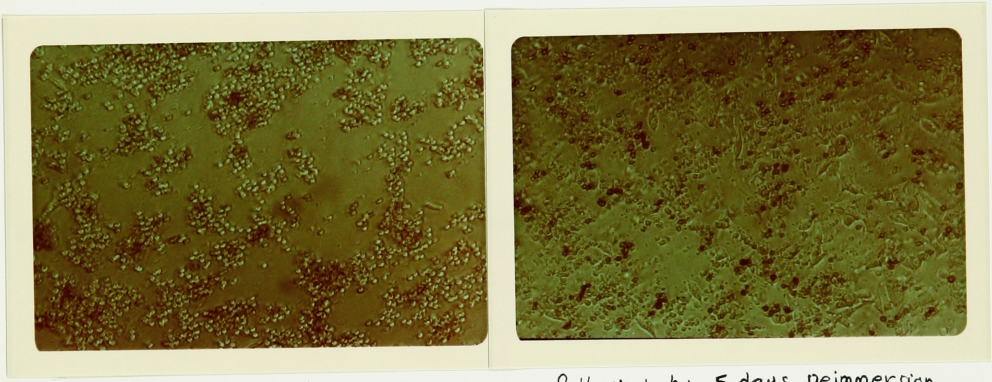
3-day exposure to calcium

Fig. 2-2

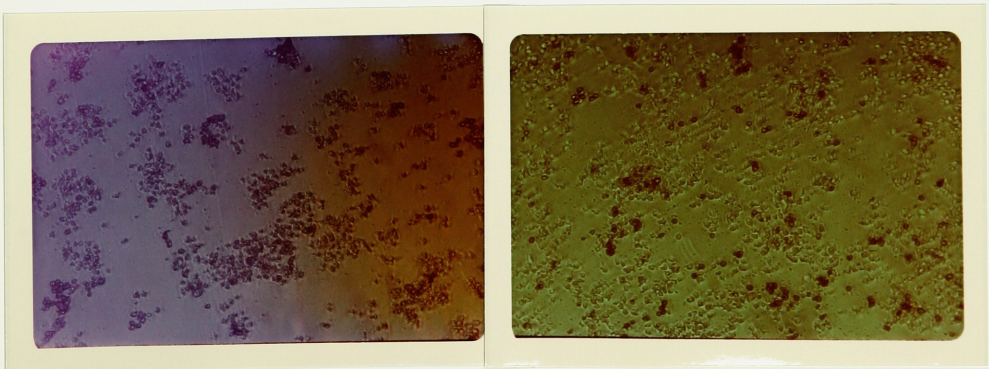
Neuroblastoma cells after varying time periods of calcium exposure.



1 day exposure to increased concentration of calcium by ten fold. → followed by 4 days of reimmersion in the normal media



2-day exposure to calcium → followed by 5 days reimmersion in the normal media

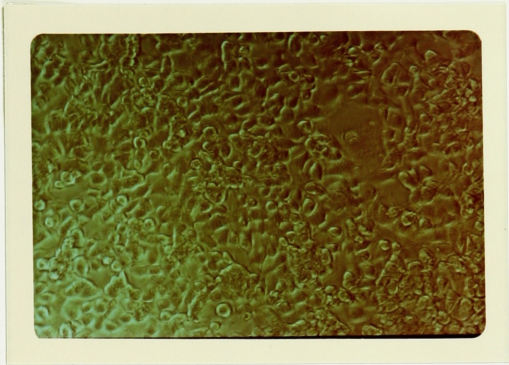


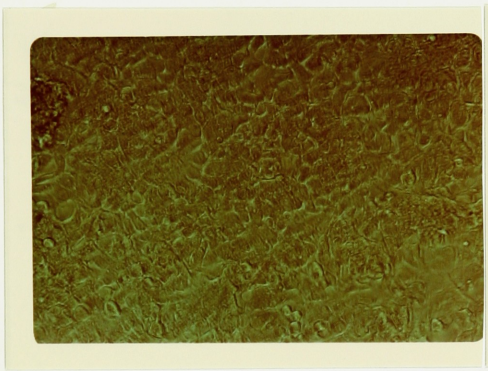
3-day exposure to calcium → followed by 4 days of reimmersion in normal media

Fig 2-3

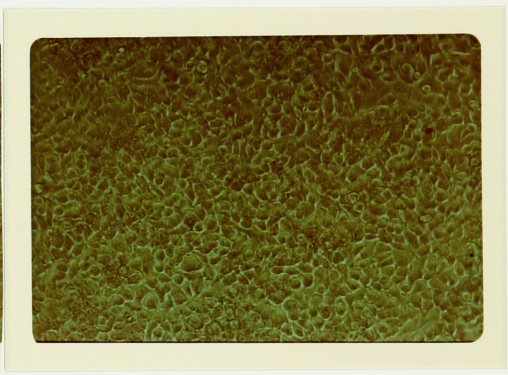
Melanoma cells after varying time periods of calcium exposure.

Normal

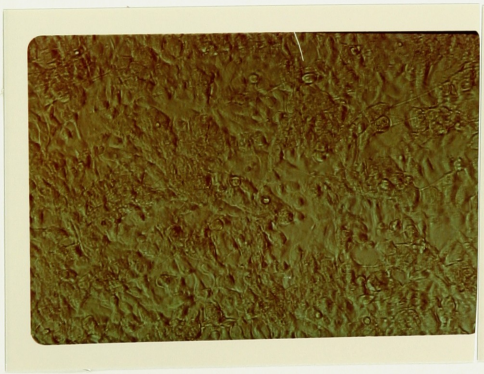




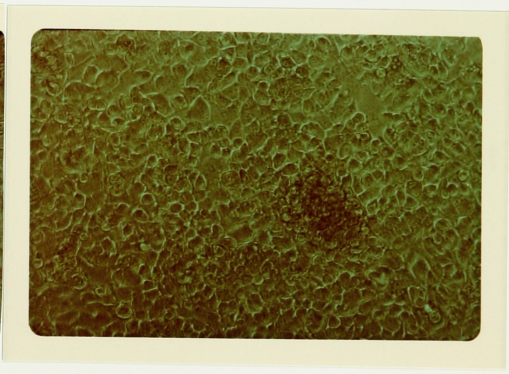
2 days exposure to increased concentration of calcium by ten-fold.



→ followed by 5 days of reimmersion in the Normal media



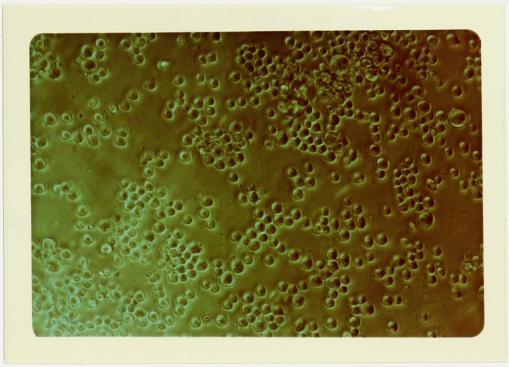
3 days exposure to calcium →



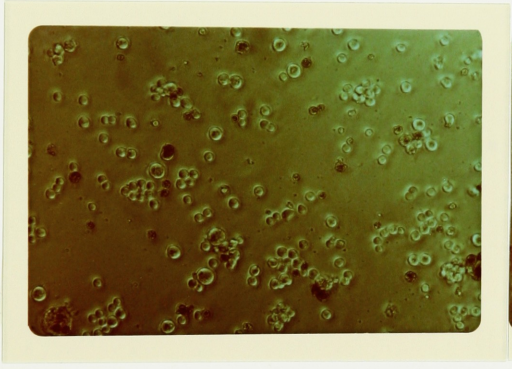
followed by 4 days of reimmersion in the Normal media Note the ability to regenerate.

Fig. 2-4.

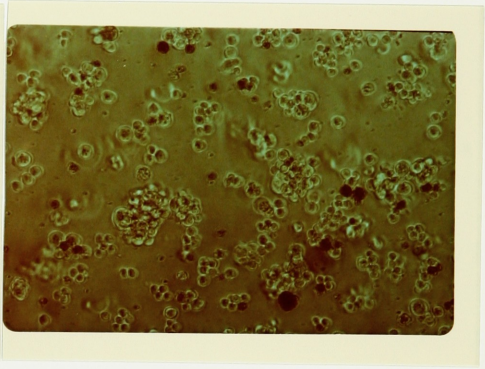
Burkitt's lymphoma RAJ-3 cells after varying time periods of calcium exposure.



Normal

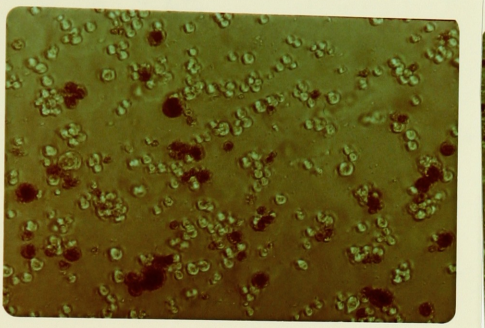


1 day exposure to increased concentration of calcium by ten fold

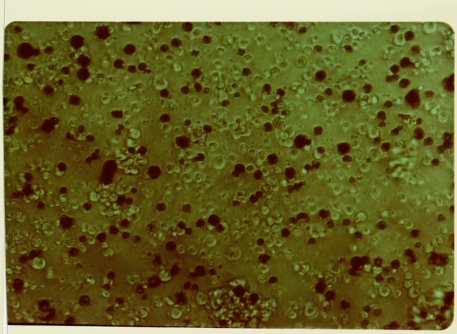


followed by 4 days reimmersion in the Normal media

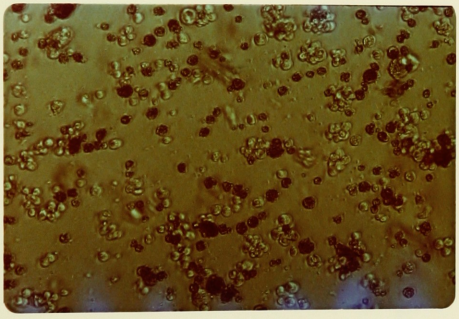
CON'T Fig. 2-4



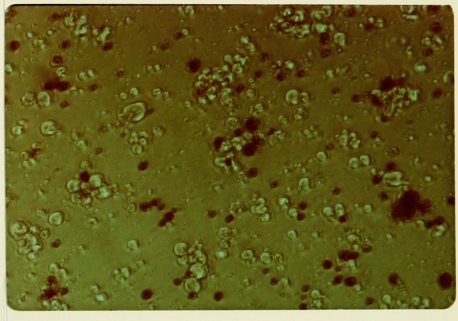
2 days exposure to increased concentration of calcium by ten-fold.



→ followed by 5 days reimmersion in the normal media



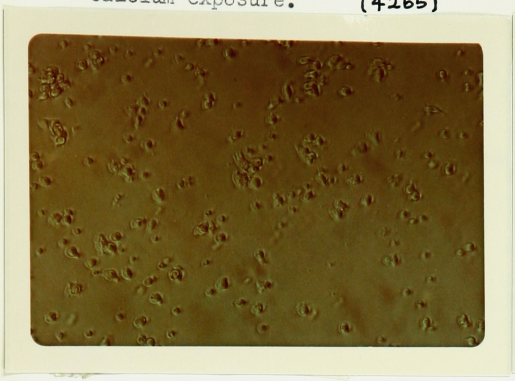
3 days exposure to increased concentration of calcium by ten-fold.



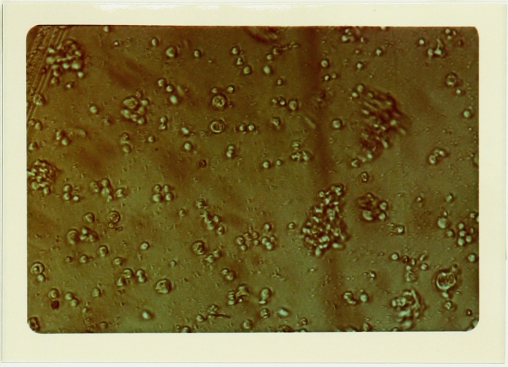
→ followed by 4 days reimmersion in the normal media

Fig. 2- 5 and 2-6

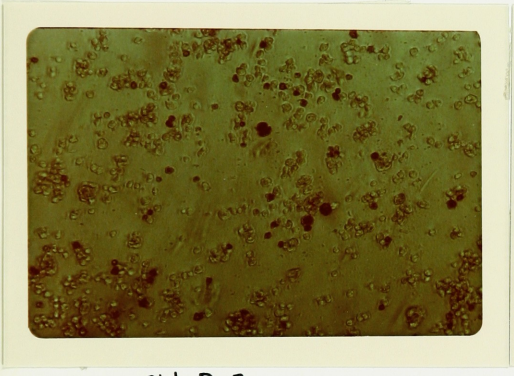
Lymphoblastic Leukemia {SKL-D / 4265} cells after varying time periods of calcium exposure.



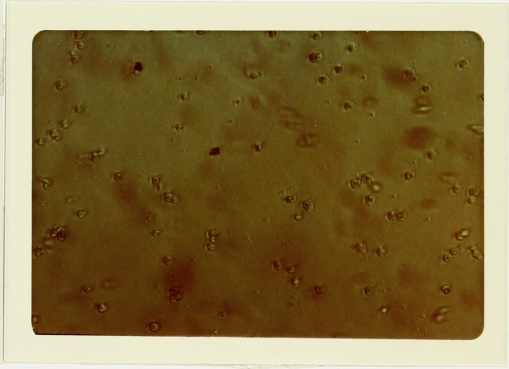
SKLD-7
6hr exposure to calcium



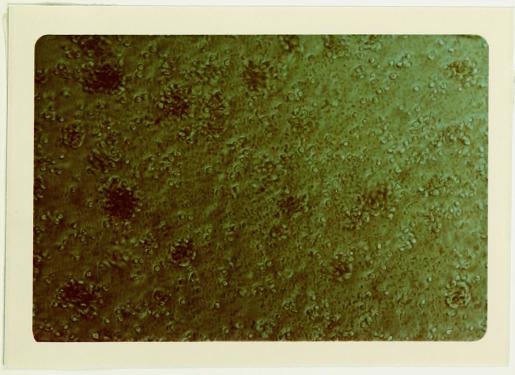
control 4265 cells



SKLD-7
22 hr exposure to calcium



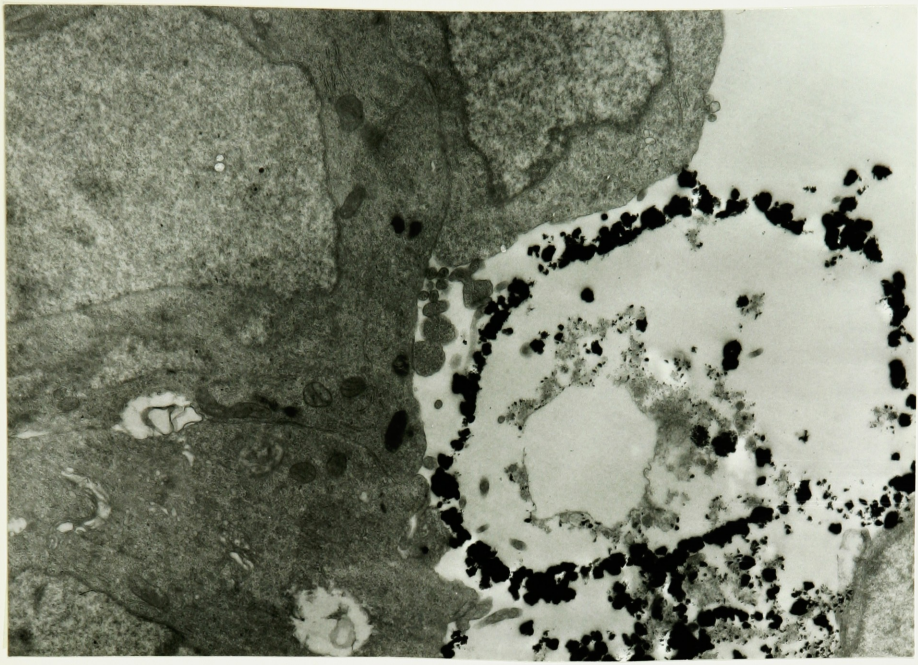
4265; 6hr exposure to calcium cells

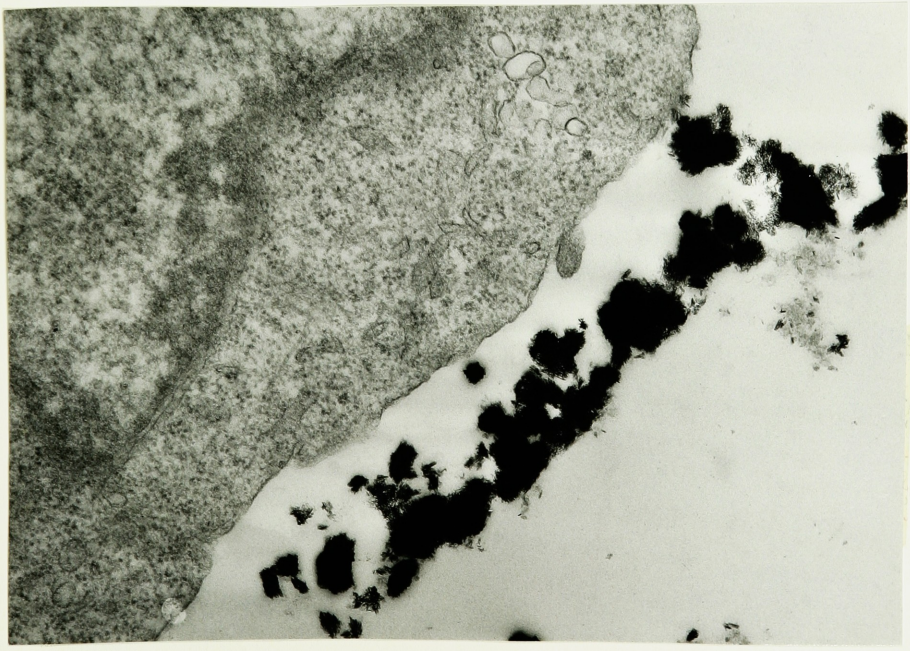


SKLD-7
3 day exposure to calcium

Fig. 2-7

Electron microscopic picture of Y-79 Retinoblastoma showing extracellular deposits.





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